UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

TESIS DOCTORAL

Análisis funcional del efecto de la nobiletina en el desarrollo embrionario bovino preimplantacional

Functional analysis of the effect of nobiletin on preimplantation bovine

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Yulia Nathaly Cajas Suárez

Directores

Dimitrios Rizos
José M. Bautista

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"No te rindas, por favor no cedas,
aunque el frio queme,
aunque el miedo muerda,
aunque el sol se esconda y se calle el viento,
aún hay fuego en tu alma,
aún hay vida en tus sueños,
porque la vida es tuya y tuyo también el deseo,
porque lo has querido y porque te quiero.

Vivir la vida y aceptar el reto,
recuperar la risa, ensayar el canto,
bajar la guardia y extender las manos,
desplegar las alas e intentar de nuevo,
celebrar la vida y retomar los cielos…"

Mario Benedetti

Yulia Natasha y Gabriela

A Angela, mamima: un ángel que me cuida desde el cielo

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List of Abbreviations

µm	micrometres
µl	microlitres
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
BME	Basal medium eagle
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic Adenosine Monophosphate
CCs	Cumulus cells
cDNA	Complementary DNA
CDK1	Cyclin-dependent kinase 1
CGs	Cortical granules
CL	Corpus luteum
CO_2	Carbon dioxide
COCs	Cumulus–oocyte complexes
Cx43	Connexin 43
CT	Cycle threshold value (qrt-PCR)
D7	Day 7
D8	Day 8
DMSO	Dymetilsulfoxhyde
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
E_2	17 beta-estradiol
EGA	Embryonic genome activation
EGF	Epidermal growth factor
et al.,	And others
FCS	Fetal calf serum
FSH	Follicle stimulating hormone
G0, G1, G2	Gaps of inactivity (cell cycle)
GCs	Granulosa cells
GSH	Glutathione
GV	Germinal vesicle

GVBD	Germinal vesicle breakdown
h	Hours
hpi	hours post insemination
HA	Hyaluronic acid
i.e.	That is
ICM	Inner cellular mass
Inh	Inhibitors III, Inhibitors IV
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
LH	Luteinizing hormone
LN_2	Liquid nitrogen
mL	millilitres
mRNA	Messenger Ribonucleic acid
M phase	Mitotic division (cell cycle)
M-I	Metaphase- I
M-II	Metaphase- II
MAPK	Mitogen-activated protein kinase
MJEGA	Major embryonic genome activation
MMLV	Moloney murine leukaemia virus
MNEGA	Minor embryonic genome activation
MPF	Maturation promoting factor
MW	Molecular weight
N/n	Number
Nob	Nobiletin
N_2	Nitrogen
O_2	Oxygen
P_4	Progesterone
PDK1	Protein-dependent kinase type 1
PIP2	Phosphatidylinositol 3,4-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PI3K	Phosphatidylinositol 3-kinase

PKA	Protein kinase A
pH	Power of hydrogen; hydrogen ion concentration
pi	post insemination
PBS	Phosphate-buffered saline
PF	Paraformaldehyde
PVP	Polyvinylpyrrolidone
RT	Reverse transcriptase
RTKs	Tyrosine kinase receptors
RT-qPCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
oSOF	Synthetic oviductal fluid
TALP	Tyrode´s albumin lactate pyruvate
TCM-199	Tissue culture medium 199
TE	Trophectoderm cells
WB	Western blotting
ZP	Zona pellucida

SUMMARY

Summary

In vitro production of embryos (IVP) is a reproductive biotechnology widely used to increase the number of offspring from superior phenotypes, to treat infertility problems and also to address fundamental questions about metabolic pathways that modulate early embryonic development. However, IVP is a multifactorial process depending on extrinsic and intrinsic factors, at both cellular and molecular levels, to increase its efficiency. Still today the IVP procedure has limitations, considering that not all oocytes have the ability to reach the blastocyst. Although a successful *in vitro* maturation (IVM) involves a nuclear, cytoplasmic and molecular maturation, necessary for fertilization and further embryo development, the blastocyst rate do not overcome 30-40% in bovine. Thus, alteration in the dynamics of early embryo development, which coincides with the switch from maternal control to embryonic genome activation (EGA) on Day 4 post fertilization, may be partially responsible for reduced embryo yield. Furthermore, it has been suggested that apart from the origin of the oocyte, the increase in reactive oxygen species (ROS) and the culture environment affecting EGA, there is also a complex network of signaling pathways responsible for cell division and differentiation and other events necessary for embryonic development, such as MAPK and PI3K/AKT.

In this context, to improve developmental rates and the quality of produced blastocysts, several studies have probed the addition of different types of natural antioxidants to the culture media. However, it is not yet clear which antioxidant is the most effective in supporting the development and quality of bovine embryos without altering the activation of signaling pathways or the EGA. Recently, an antioxidant identified from citrus peel called nobiletin has attracted more attention as it is easily absorbed through cytoplasmic membranes due to its structure and lipophilic nature, while has a wide spectrum of biological activities including antioxidant functions and cell cycle regulation.

Therefore, the objective of the first experiment of this doctoral thesis was to evaluate the antioxidant role of nobiletin during IVM on matured bovine oocyte quality and their developmental competence, as well as quantitative changes on gene expression in oocytes, their cumulus cells and resulting blastocysts. The results revealed that 25 and 50 μM nobiletin supplementation increased the percentage of oocytes reaching metaphase II, as well as cleavage rate and cumulative blastocyst yield on Days 7 and 8. Also, oocytes matured with 25 and 50 μM nobiletin showed a higher rate of cortical granules migration and mitochondrial activity and a reduction in ROS and glutathione (GSH) levels. This was linked to a modulation in the expression of *CYP51A1*, *GJA1*, *BCL2*, *BMP15*, *MAPK1*, *SOD2* and *CLIC1* genes. Thus, we concluded that nobiletin

offers a novel alternative for counteracting the effects of the increase in ROS production during IVM, improves oocyte nuclear and cytoplasmic maturation, and subsequent embryo development and quality in cattle.

Based on these positive results of nobiletin during IVM and with evidence in cell line studies shown that nobiletin interact with several signalling pathways (ERK, PI3K/AKT, CREB) to promote survival, in the second experiment of this doctoral thesis we aimed to evaluate whether nobiletin supplementation during the minor (2-8 cell stage; MNEGA) or major (8-16 cell stage; MJEGA) phase of EGA improves embryonic development and blastocyst quality and if it affects PI3K/AKT signaling pathway. The results revealed that 5 and 10 μM nobiletin increased the cumulative blastocyst yield on Day 7 and 8 and their mitochondrial activity, irrespective of EGA phase. Besides, mRNA abundance of *CDK2*, *H3-3B*, *H3-3A*, *GPX1*, *NFE2L2* and *PPARα* transcripts were increased in 8-cells, 16-cells and blastocysts produced with nobiletin supplementation during MNEGA and MJEGA, and the immunofluorescence analysis revealed immunoreactive proteins for p-AKT forms (Thr308 and Ser473) in bovine blastocysts. Therefore, we concluded that nobiletin supplementation during EGA has a positive effect on preimplantation bovine embryonic development *in vitro* and corroborates with the quality improvement of produced blastocysts, which could be modulated by the activation of AKT signaling pathway.

In view of the results related with the immunoreactive proteins for p-AKT forms (Thr308 and Ser473) in bovine blastocysts, in the third experiment of this doctoral thesis we aimed to establish whether, when the AKT cascade is inhibited using inhibitors III or IV, nobiletin supplementation to the *in vitro* culture (IVC) media during the MNEGA or MJEGA phase is able to modulate the development and quality of bovine embryos. The results showed that inhibition of AKT signaling pathway in early bovine embryos reduced their developmental capacity. However, nobiletin supplementation to the *in vitro* embryo culture medium during the two main phases of EGA recovered the adverse effects of AKT signaling inhibition on embryo developmental competence and their quality by increasing blastocysts cell number and modulating the expression of *CDX2*, *GPX1*, *NFE2L2*, *POU5F1*, and *FOSL1* transcripts in early-stage embryos. Besides, the rpS6 phosphorylation level on 8-cell (MNEGA) and 16-cell (MJEGA) stage embryos was significantly lower in both AKT inhibitor treatments. Nevertheless, nobiletin was able to partially recover this adverse effect showing significantly higher rpS6 phosphorylation level. In conclusion, nobiletin supplementation in presence of AKT inhibitors counteracts their adverse effects on developmental competence and embryo quality by increasing the total, trophectoderm (TE) and inner cell mass (ICM) cells and modifies the relative abundance of keys genes linked to cell proliferation and differentiation. Moreover, increase of rpS6 phosphorylation level by

nobiletin, whether or not AKT inhibitors were present, indicates that nobiletin probably uses another signaling system besides PI3K/AKT pathway during early embryo development in bovine.

This research investigated for the first time the effect of nobiletin supplementation, a natural antioxidant, during IVM and specific phases of IVC related with EGA on bovine embryo development and quality, as well as its possible interaction with the AKT signaling pathway. The benefits of nobiletin found can be attributed to its bioactivity, chemical structure, and antioxidant properties, and might be a tool to overcome ROS disorders in bovine IVP embryos and furthermore to improve assisted reproductive technologies in mammals. Nevertheless, future studies are necessary to establish the specific receptors and pathways that nobiletin modulates during *in vitro* embryonic development.

RESUMEN

Resumen

La producción *in vitro* de embriones (IVP) es una biotecnología reproductiva ampliamente utilizada para aumentar el número de descendientes de fenotipos superiores, para tratar problemas de infertilidad y también para abordar preguntas fundamentales sobre las vías metabólicas que modulan el desarrollo embrionario temprano. Sin embargo, la IVP es un proceso multifactorial que depende de factores extrínsecos e intrínsecos, tanto a nivel celular como molecular, para aumentar su eficacia. Aún hoy, la IVP tiene limitaciones, considerando que no todos los ovocitos tienen la capacidad de llegar a la etapa de blastocisto. Aunque una maduración *in vitro* (IVM) exitosa implica una maduración nuclear, citoplasmática y molecular, necesaria para la fecundación y el desarrollo posterior del embrión, la tasa de blastocistos no supera el 30-40% en bovinos. Por lo tanto, la alteración en la dinámica del desarrollo embrionario temprano, que coincide con el cambio del control materno a la activación del genoma embrionario (EGA) en el día 4 posterior a la fecundación, puede ser parcialmente responsable de la reducción del rendimiento embrionario. Se ha sugerido que además del origen del ovocito, el aumento de especies reactivas de oxígeno (ROS) y el ambiente de cultivo que afecta a EGA, existe una compleja red de vías de señalización responsables de la división y diferenciación celular y otros eventos necesarios para el desarrollo embrionario, como las cascadas genéticas MAPK y PI3K/AKT.

En este contexto, para mejorar las tasas de desarrollo y la calidad de los blastocistos producidos, varios estudios han investigado la suplementación de diferentes tipos de antioxidantes naturales a los medios de cultivo. Sin embargo, aún no está claro qué antioxidante es el más eficaz para apoyar el desarrollo y la calidad de los embriones bovinos sin alterar la activación de las vías de señalización o la EGA. Recientemente, un antioxidante identificado a partir de la cáscara de los cítricos llamado nobiletina ha atraído más atención ya que se absorbe fácilmente a través de las membranas citoplasmáticas debido a su estructura y naturaleza lipofílica, y tiene un amplio espectro de actividades biológicas que incluyen funciones antioxidantes y regulación del ciclo celular.

Por lo tanto, el objetivo del primer experimento de esta tesis doctoral fue evaluar el papel antioxidante de la nobiletina durante la IVM en la calidad de los ovocitos bovinos madurados y su competencia para desarrollarse, así como los cambios cuantitativos de la expresión génica en los ovocitos, las células del cúmulo que lo rodean y los blastocistos resultantes. Los resultados revelaron que la suplementación con 25 y 50 μM de nobiletina, aumentó el porcentaje de ovocitos que alcanzaron la metafase II, así como la tasa de división y el rendimiento acumulativo de los blastocistos en los días 7 y 8. Además, los ovocitos madurados con 25 y 50 μM de nobiletina

mostraron una mayor tasa de migración de gránulos corticales, un incremento de la actividad mitocondrial y una reducción en los niveles de ROS y GSH. Esto se relacionó también con una modulación en la expresión de los genes *CYP51A1*, *GJA1*, *BCL2*, *BMP15*, *MAPK1*, *SOD2* y *CLIC1*. Por lo tanto, concluimos que la nobiletina ofrece una alternativa novedosa para contrarrestar los efectos del aumento de la producción de ROS durante la IVM, y mejorar la maduración nuclear y citoplasmática de los ovocitos y el posterior desarrollo y calidad embrionaria en el bovino.

Basándonos en el efecto positivo de la nobiletina durante la IVM y en estudios con líneas celulares que evidencian que la nobiletina interactúa con varias vías de señalización (ERK, PI3K/AKT, CREB) para promover la supervivencia; en el segundo experimento de esta tesis doctoral nos propusimos evaluar si la suplementación con nobiletina durante la fase menor (estadio de células 2-8; MNEGA) o mayor (estadio de células 8-16; MJEGA) de EGA mejora el desarrollo embrionario y la calidad del blastocisto y si afecta la vía de señalización PI3K/AKT. Los resultados revelaron que concentraciones de 5 y 10 µM de nobiletina aumentaban el rendimiento acumulativo de blastocistos en los días 7 y 8 y su actividad mitocondrial, independientemente de la fase de EGA analizada. Además, la abundancia de ARNm de los transcritos de *CDK2*, *H3-3B*, *H3-3A*, *GPX1*, *NFE2L2* y *PPARα* se incrementó en los estadios de 8, 16 células y blastocistos producidos con la adición de nobiletina durante la MNEGA y MJEGA, y el análisis de inmunofluorescencia reveló proteínas inmunorreactivas para la forma p-AKT (Thr308 y Ser473) en los blastocistos bovinos. Por lo tanto, concluimos que la suplementación con nobiletina durante la EGA tiene un efecto positivo en el desarrollo embrionario bovino preimplantacional *in vitro,* corroborado con la mejora de la calidad de los blastocistos producidos, y podría ser modulada por la activación de la vía de señalización AKT.

Con la importancia de las proteínas inmunorreactivas para las formas de p-AKT (Thr308 y Ser473) en los blastocistos bovinos, en el tercer experimento de esta tesis doctoral nos propusimos establecer si, tras la inhibición de la cascada de AKT mediante los inhibidores III o IV; la suplementación con nobiletina al medio IVC durante la fase MNEGA o MJEGA es capaz de modular el desarrollo y la calidad de los embriones bovinos. Los resultados revelaron que la inhibición de la vía de señalización de AKT en embriones bovinos tempranos reducía su capacidad de desarrollo. Sin embargo, la suplementación con nobiletina en el medio de cultivo de embriones *in vitro* durante las dos fases principales de EGA recuperó los efectos adversos de la inhibición de la señalización de AKT sobre el desarrollo embrionario y su calidad al aumentar el número de células de los blastocistos y modular la expresión de *CDX2*, *GPX1*, *NFE2L2*, *POU5F1* y *FOSL1* en embriones tempranos. Además, el nivel de fosforilación de rpS6 en los embriones en etapa de 8 células (MNEGA) y 16 células (MJEGA) fue significativamente menor en ambos tratamientos con

inhibidores de AKT. Sin embargo, la nobiletina fue capaz de recuperar parcialmente este efecto adverso mostrando un nivel de fosforilación de rpS6 significativamente mayor. En conclusión, la suplementación con nobiletina en presencia de inhibidores de AKT contrarresta sus efectos adversos sobre la capacidad de desarrollo y la calidad del embrión al aumentar el número de células totales, del trofectodermo (TE) y de la masa celular interna (ICM) y modifica la abundancia relativa de genes cruciales para la proliferación y diferenciación celular. Además, el aumento de la nobiletina en el nivel de fosforilación de rpS6, en presencia o ausencia de inhibidores de AKT, indica que la nobiletina probablemente usa otra ruta de señalización además de la vía PI3K/AKT durante el desarrollo embrionario temprano en bovinos.

Este es el primer estudio que ha investigado el efecto de la suplementación con nobiletina, un antioxidante natural, durante la IVM y las fases específicas del IVC relacionadas con la EGA sobre el desarrollo y la calidad del embrión bovino, así como su posible interacción con la vía de señalización de AKT. Los efectos beneficiosos demostrados de la nobiletina, se pueden atribuir a su bioactividad, estructura química y propiedades antioxidantes, y podrían ser una herramienta para superar los trastornos de ROS en los embriones bovinos IVP y mejorar las tecnologías de reproducción asistida en mamíferos. Sin embargo, son necesarios estudios futuros para establecer los receptores específicos y las cascadas genéticas que la nobiletina modula durante el desarrollo embrionario *in vitro*.

INTRODUCTION

1. PREIMPLANTATION EMBRYO DEVELOPMENT

1.1. Periconception environment *in vivo*

The generation of mature oocyte includes primordial follicle recruitment, granulosa/theca cell proliferation, oocyte maturation and ovulation. Since birth, follicular growth occurs in a wave-like pattern: recruitment, selection, dominance and atresia (Driancourt, 2001). In cattle, during oogenesis, which is a process that leads to the formation of the oocyte (Fair, 2003), oogonia undergoes mitotic divisions until it stops at the diplotene stage of prophase I (the germinal vesicle stage (GV)) (Picton *et al.,* 1998), and all the process takes place within structures known as follicles, which are localized in the ovarian cortex. As the oocyte progresses through oogenesis within the follicle, the shape of the granulosa cells (GCs) change, increase and proliferate until finish forming a tertiary follicle, also called antral follicle that consists of three distinct layers (theca externa at the periphery of the follicle, theca interna and mural granulosa cell layer), a cavity (antrum) filled with follicular fluid and the oocyte which lies within it surrounded by the GCs (Fair, 2003). The bovine oocyte reaches its full size when the follicle enclosing it reaches a diameter of approximately 3 mm (Fair *et al.,* 1995). Follicular growth takes place in follicular waves (Ireland, 1987; Roche, 1996), each one starting with the recruitment of a cohort of follicles followed by a process of selection, dominance and atresia (Driancourt, 2001) and is regulated by the interaction of gonadotropins from the pituitary gland (i.e. follicle stimulating hormone (FSH) and luteinizing hormone (LH)) and proteins/peptides from the ovaries (Figure 1).

Oocyte maturation is elicited by the LH peak and stage ends once the maximum size in the follicle is reached, just a few hours before ovulation, at which time the stimulation by LH allows the oocyte to resume meiosis and the rupture of the germinal vesicle (GV), known as germinal vesicle breakdown (GVBD), takes place (Picton *et al.,* 1998; Smith, 2001).

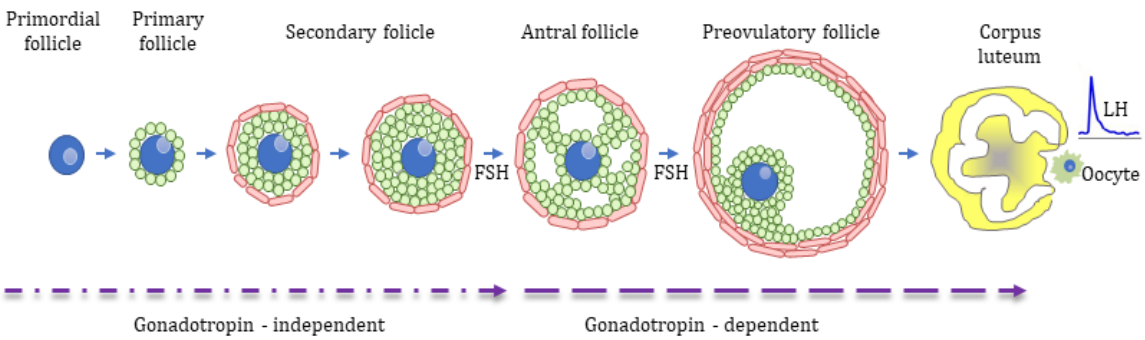

Figure 1. Summary of the main stages of folliculogenesis. Folliculogenesis begins with the activation of the primordial follicles that are subsequently recruited to become primary follicles. GCs proliferate and form several layers around the oocyte. From this stage, a layer of theca cells surrounds the follicle, and it begin to produce androgens, which convert to estrogens in GCs. The early stages of follicular growth to secondary

are independent of gonadotropins, but the progression beyond this stage is strictly dependent on FSH stimulation. Ovulation eventually occurs, triggered by a LH preovulatory spike. Adapted from Driancourt, 2001.

Oocyte maturation involves: (i) nuclear maturation, characterized by chromosome condensation and expulsion of the first polar body, which is vital to generate a competent oocyte (Palma *et al.,* 2012); (ii) cytoplasmic maturation that involves cytoskeleton dynamics and rearrangement, organelles redistribution, cortical granules (CGs) migration to the periphery of the oocyte (they will contribute to the block of polyspermy after fertilization) (Figure 2) and (iii) molecular maturation that consists of the transcription and processing of maternal mRNA which is stored in a stable inactive form until translational recruitment, necessary for subsequent development (during the development of the early embryo) (Ferreira *et al.,* 2009); moreover the proteins derived from these mRNAs are involved in maturation, fertilization, pronuclear formation and early embryogenesis.

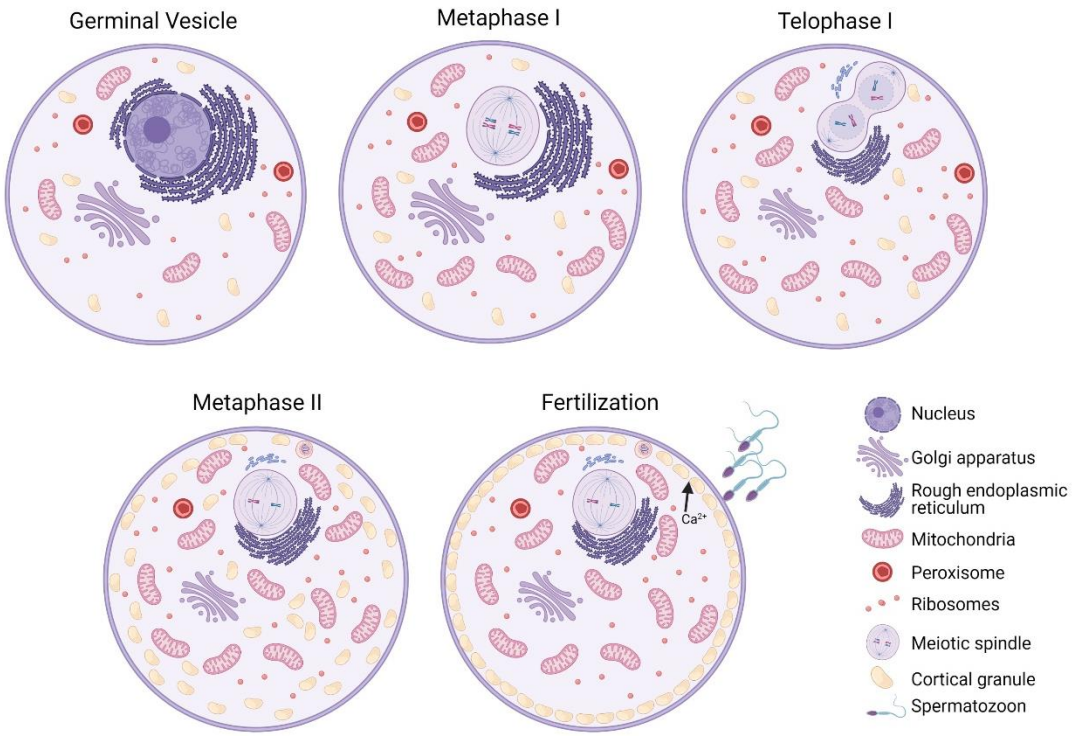

Figure 2. Schematic overview of the distribution of cytoplasmic organelles during maturation and fertilization. Figure adapted from Ferreira *et al.,* 2009 and created with BioRender.com

The regulation of the cyclic Adenosine Monophosphate (cAMP) levels is crucial for the control of the meiotic cycle, as several studies have shown high levels of cAMP in the ooplasm inhibit the progression of meiosis, while low levels activate it (Sirard *et al.,* 1998; Webb *et al.,* 2002). This regulation is controlled by: Gap junctions, through which GCs transfer cAMP to

ooplasm; the internal production of cAMP in ooplasm, which transforms Adenosine Triphosphate (ATP) into cAMP, and the activation of phosphodiesterase (PDE) that inactivates the cAMP dependent Protein kinase A (PKA), one of the main regulatory proteins of the maturation promoting factor (MPF) cascade (Kalinowski *et al.,* 2004). Normally, it is assumed that the decrease in cAMP is caused by the preovulatory LH surge, which modulates the closure of the gap junctions. This event has been related to the phosphorylation of Connexin 43 (Cx43) by a PhosphatidylInositol-3-Kinase (PI3K), which leads to the closure of the communication of the gap junction complex, and therefore decreases the supply of cAMP (Sun & Nagai, 2003). It has been postulated that the phosphorylation of MPF is what finally triggers the restart of the meiotic cycle. MPF consists of a catalytic subunit the cyclin-dependent kinase 1 (CDK1), and a regulatory subunit Cyclin B (CYCB) (Adhikari & Liu, 2014). The oocyte accumulates the CDK1 and CYCB subunits during folliculogenesis in the form of a pre-MPF complex. For meiosis to resume, the pre-MPF complex is activated synchronously with the GVBD. For this, CDK1 must be dephosphorylated to allow CYB binding and form the active MPF (Adhikari & Liu, 2014). This will lead to the disintegration of the nuclear membrane in the GVBD, the condensation of chromosomes and the appearance of microtubules within the nuclear zone to form the metaphase plate, a characteristic sign of meiosis resumption. Finally, nuclear maturation must end with the migration of the metaphase plate to the periphery of the oocyte and the expulsion of a set of chromosomes in the form of a polar body, generating an oocyte with a set of haploid chromosomes ready to be fertilized (Sathananthan *et al.,* 2006)(Figure 3).

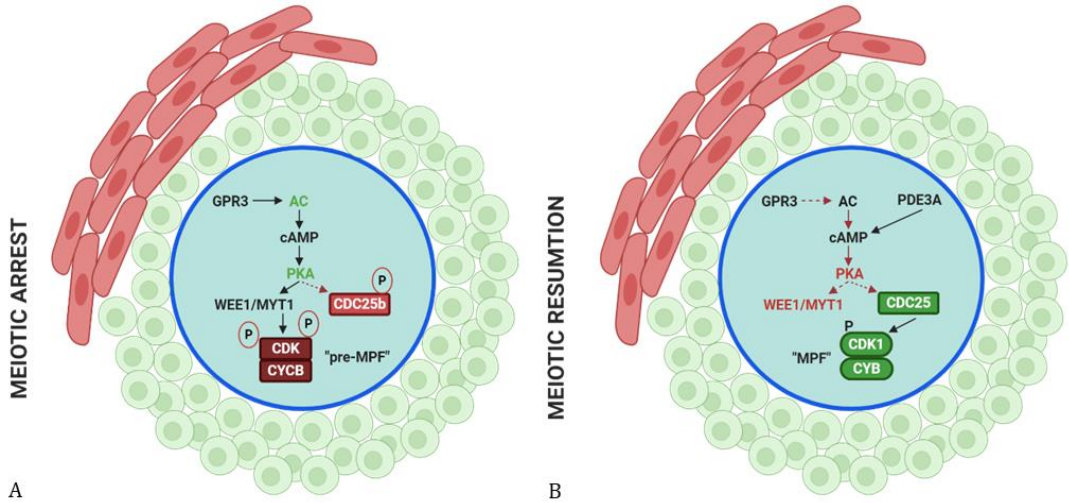

Figure 3. Signaling pathways to trigger the restart of meiosis in oocytes. (A). Signaling that maintains high amounts of cAMP to keep the oocyte in meiotic arrest. (B). Meiosis activation pathways that decrease the amount of cAMP. The red lines represent inhibition and the black lines activation. Proteins in green represent activated proteins and proteins in red inhibited in the classic activation pathway for the restart of meiosis. Gs-linked receptor (GPR3) activated, stimulates adenylyl cyclase (AC) to cause an elevation of cAMP and activates protein kinase A (PKA), which causes the cell cycle regulatory complex, CDK1/cyclin B (CYB), to be phosphorylated and thereby inactivated. Figure modified from Mehlmann, 2005 and created with BioRender.com

Other important parameters of oocyte maturation quality are CGs migration to the periphery of the oocyte, that contributes to the block of polyspermy after fertilization, and the secretion of hyaluronic acid (HA) by cumulus cells (CCs), which causes the spaces between the CCs to enlarge and allows the embedding of granulose cells in a sticky, mucified matrix (Eppig, 2001), a process denominated cumulus expansion. After the onset of the LH surge, ovulation takes place, and the now-matured oocyte (arrested in metaphase II (M-II)) is released along with its surrounding cells (CCs), and the fimbria of the infundibulum allows the passage of the cumulus-oocyte complex (COC) into the oviduct.

After copulation, the spermatozoa suffer a sequence of biochemical changes leading to destabilization of the cell membrane, enabling the acrosome reaction in response to contact with the zona pellucida (ZP). These biochemical changes are called capacitation, it starts with removal of seminal plasma proteins by glycosaminoglycans present in the uterus and finishes in a characteristic increase in sperm motility called hyperactivation. During natural mating, semen is deposited in the anterior vagina and travels through the cervix and the uterus to the site of fertilization in the oviduct. During its transit through the female tract, complex mechanisms will influence sperm transport, immunological tolerance to sperm and sperm selection (Machaty *et al.,* 2012). To achieve successful fertilization, the male gamete needs to be capacitated while the female gamete must be matured. For fertilization to take place, firstly, the sperm must penetrate through the CCs to reach the ZP. The sperm binds to the ZP and this interaction elicits the acrosome reaction (which involves the fusion and extensive vesiculation of sperm plasma and outer acrosomal membranes) (Bleil & Wassarman, 1983). Once the sperm is in the perivitelline space, the sperm and oocyte membranes fuse. Following fusion of sperm and oocyte, the CGs in the oocyte release their contents into the perivitelline space (cortical reaction), causing the ZP to become refractory to sperm binding (Sun & Nagai, 2003), thus contributing to the block of polyspermy. The sperm penetration elicits meiotic resumption in the oocyte (which was arrested in M-II) followed by the extrusion of the second polar body.

Finally, the male and female pronuclei fuse in a process termed syngamy resulting in zygote formation. After fertilization, the zygote starts to divide in a series of mitotic divisions without prior cell growth, giving rise to the embryonic cells, termed blastomeres. The first cleavage takes place around 20h after the estimated time of ovulation (Lonergan *et al.,* 1999) and embryo cleavages are supported by the mRNA and proteins stored in the oocyte during oogenesis (Memili & First, 2000). Studies indicate that a change from maternal to embryonic control occurs during early embryonic development, in a process called embryonic genome activation (EGA) (Gad *et al.,* 2012; Graf *et al.,* 2014). At the start of early embryogenesis, all mRNAs and proteins are of maternal origin and as development progresses, these reserves gradually degrade while

embryonic transcripts are synthesized (Tadros & Lipshitz, 2009). The EGA occurs in distinct waves, which are species-specific, in the bovine embryo is characterized by two different forms: (i) minor EGA (MNEGA) (2-cell to 8-cell stage) where zygotes and early embryos are transcriptionally and translationally active; (ii) major EGA (MJEGA) (8- cell to 16-cells stage) which includes a gradual degradation of mRNA molecules of maternal origin, and activation of transcription of embryonic genome, together with a change in the protein synthesis, and these events are key factors for successful embryonic development and differentiation (Gad *et al.,* 2012) (Figure 4).

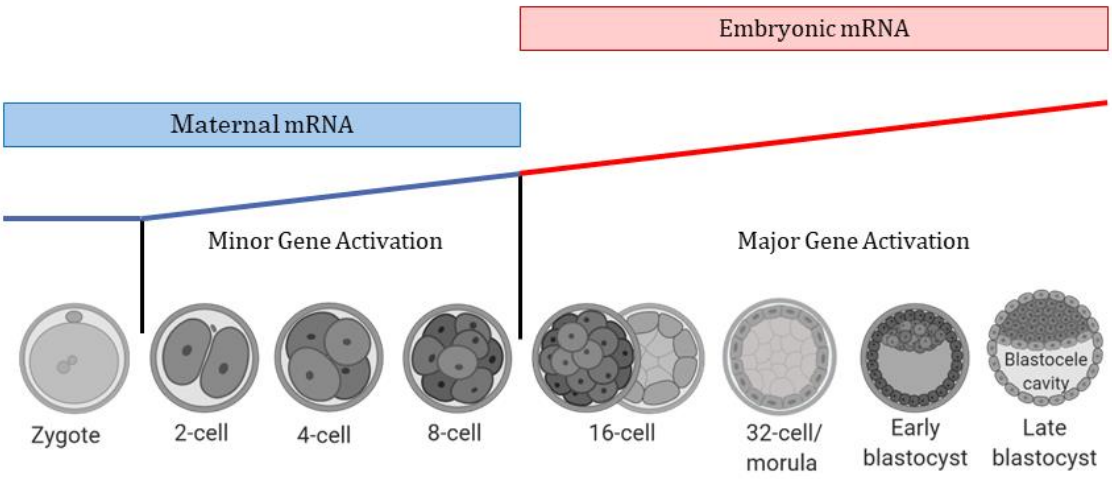

Figure 4. Development phases of bovine embryo. Cleavage, compact morula stage and differentiation into TE and ICM at the blastocyst stage. Embryonic gene expression and embryonic protein synthesis depends mainly on the maternal transcripts (mRNA and proteins) stored in the oocyte. MNEGA occurs between the 2-8-cell stage and MJEGA starts at the 8-cell stage. Figure adapted and modified from Badr *et al.,* 2007; Memili & First, 2000 and created with BioRender.com

Bovine embryo remains in the oviduct until the 16-cell stage (Day 4 of pregnancy) where the described key events (the first cleavages and EGA) take place and then it enters the uterus where compaction of the morula occurs (begins at the 32-cell stage), blastocyst formation (64 - to 128- cell stage) and elongation (Hackett *et al.,* 1993; Van Soom *et al.,* 1992). During the morula stage, two distinct cellular populations begin to separate: the inner and the outer cells. Because of the nature of the tight junctions (outer cells) and the gap junctions (inner cells) that allows the establishment of an intercellular communication, the embryo forms two different cell populations known as TE (outer cells) and ICM. ICM is defined as a group of cells located eccentrically within the compact morula/blastocyst which, after further differentiation, gives rise to the fetus (Van Soom *et al.,* 1997), while the outer cells possess apical tight junctions which play part in the formation of the blastocoel (cavity filled with fluid) and furthermore give rise to the placental membranes. This compaction in the morula is an important morphogenetic event of development

increasing cell-to-cell contact between blastomeres where adherent's junctions composed of cadherins are essential (Watson & Barcroft, 2001).

A blastocyst contains between 64 - 128 cells and it is still surrounded by the ZP (Fleming *et al.,* 2000; Lonergan *et al.,* 2016). The entrance of fluid into the blastocoel elicits blastocyst expansion, with the embryo occupying all the perivitelline space. An expanded blastocyst contains about 160 cells. On Days 9-11 post-fertilization, the ZP begins to fragment and the blastocyst 'hatches' due to mechanical forces exerted by blastocyst expansion and the production of proteolytic enzymes by the trophoblast (Wolf *et al.,* 2003). In this stage, the blastocyst develops into an ovoid form and then elongates to form a filamentous conceptus on Day 16-17, during which time it secretes increasing quantities of interferon tau for maternal pregnancy recognition and occupies the entire length of the uterine horn (Clemente *et al.,* 2009; Hue *et al.,* 2012). During conceptus elongation, the progesterone (P_4) synthesized by the corpus luteum (CL) acts indirectly via the endometrium to stimulate embryonic growth (Clemente *et al.,* 2009). After Day 19, the elongated conceptus begins implantation and attachment of the TE to the endometrial luminal epithelium (Guillomot, 1995).

1.2. Components and signaling pathways important in maturation and embryonic development

During oocyte maturation and early embryo development several signaling pathways are involved in the complex developmental processes related with assorted biological actions such as cell division, growth, differentiation, migration and expression of specific genes related to cell polarity and differentiation of the outer cells of the early embryo. These pathways include mitogen-activated protein kinase (MAPK), PI3K/AKT, bone morphogenetic proteins (BMPs), signal transducer and activator of transcription (STAT) and Janus-activated-kinase (JAK) (Gad *et al.,* 2012; Zhang *et al.,* 2007) (Figure 5).

1.2.1. Role of the MAPK pathway during oocyte maturation and embryonic development

For all these phases to develop, it is important to know which are the main signaling cascades during the restart of meiosis that help trigger and regulate oocyte maturation and embryo developmental process, one of them being the MAPK pathway. In oocytes, one of the first events associated with the stimulation of the LH and FSH receptors is precisely the activation of the MAPK cascade (Sela-Abramovich *et al.,* 2005). MAPK pathway transmits extracellular signals to intracellular targets and mediates several cellular processes including cell proliferation, growth and differentiation (Plotnikov *et al.,* 2011). Interestingly, studies of oocyte proteome at different stages revealed that the MAPK pathway is the most expressed during M-II, where it is

believed to exert an important regulation to give the second meiotic arrest (Wang *et al.,* 2010). During oocyte maturation, MAPK activates the Mos protein that plays a key regulatory role of the cell cycle during meiosis and is necessary for the activation of the MPF (Zhang & Liu, 2002). Preimplantation embryos use a MAPK pathway to relay signals from external environment in order to prepare appropriate responses and adaptations to a changing milieu. p38 MAPK in the early embryo is a regulator of filamentous actin in the 8-cell stage embryo and is an important regulator of compaction and development of the embryo (Natale *et al.,* 2004).

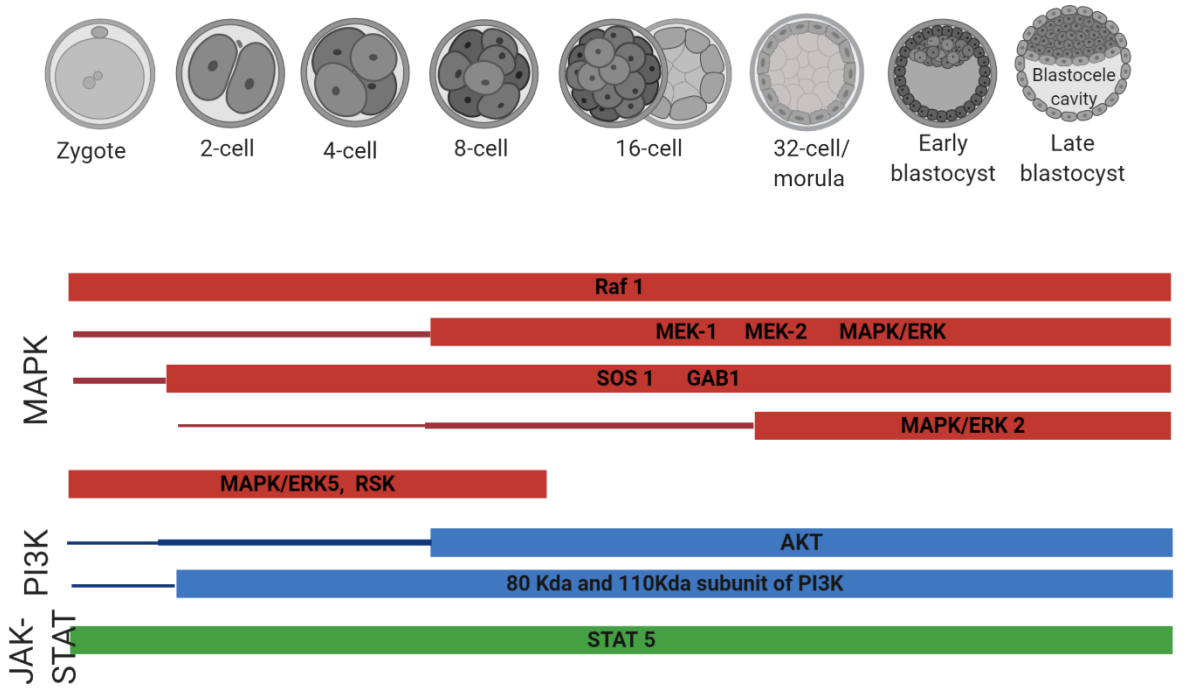

Figure 5. Stage-specific expression of several signal-transduction pathways in the preimplantation development of the mammalian embryo. Red, mitogen-activated protein kinase (MAPK); blue, phosphatidylinositol 3-kinase (PI3K); green, JAK-STAT signaling pathway (Zhang *et al.,* 2007). Figure created with BioRender.com

Also, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), which represent one of the best characterized proteins within MAPK signaling, play an important role in signaling cascades that lead to the acquisition of nuclear competence by binding directly to DNA and acting as transcriptional repressors of several cytokine-induced genes (Plotnikov *et al.,* 2011). The substrates of ERK 1/2 pathway are localized in several cellular compartments, cytoplasmic organelles, membranes and nuclear proteins and those that participate in the regulation of steroidogenesis, in the survival and proliferation of GCs, in the restart of meiosis and microtubule dynamics (Sasseville *et al.,* 2010). In adherent junctions, sites of cell-cell and cell-matrix contact, ERK1/2 activation induces the translocation to the nucleus where it accesses nuclear transcription factors and proteins that are then phosphorylated to bring relevant changes in gene

expression (Raman *et al.,* 2007). ERK pathway can link G0/G1 mitogenic signals by association with cyclin-dependent kinases (E, A and Cdk2). Cdks are activated by Cdc25 phosphatases that are directly phosphorylated and activated by the c-Raf-1 kinase. Ras/Raf signaling is involved in the induction of c-myc expression, which is a DNA binding protein that control expression of genes for cell proliferation (Zhang & Liu, 2002). Activation of ERK1/2 requires phosphorylation of threonine and tyrosine residues that is carried out by the upstream activator kinase, and their stimulation change the localization of transcription regulators and cytoskeletal proteins (Sasseville *et al.,* 2010).

1.2.2. Role of the PI3K pathway during oocyte maturation and embryonic development

The PI3K signaling pathway consists of several molecules, including kinases, phosphatases and transcription factors that are fundamental in processes such as cell proliferation, survival, migration and metabolism (Plotnikov *et al.,* 2011). The PI3Ks are activated through binding of Src homology (SH2) domain in the adaptor subunit to autophosphorylated tyrosine kinase receptors and are divided into three classes (class I, class II and class III). The class I group has been associated with reproductive physiology and is activated by tyrosine kinase receptors (RTKs) such as epidermal growth factor receptor or gonadotropin-releasing hormone receptor, such as FSHr and LHr (Vanhaesebroeck *et al.,* 2010). PI3K phosphorylates the hydroxyl groups of phosphatidylinositol 3,4-bisphosphate (PIP2) to convert it into phosphatidylinositol 3,4,5-triphosphate (PIP3) which is a second messenger for the activation of the different components of the pathway. The components of the PI3K pathway include protein kinase B better known as AKT, protein-dependent kinase type 1 (PDK1) and the forkhead family of transcription factors such as FOXO (Liu *et al.,* 2007).

AKT is the key molecule of the PI3K pathway, and its activation begins by the stimulation of RTKs or G-protein couple receptor (GPCR) leading to plasma membrane recruitment and activation of one or more isoforms of the class I PI3K family (Vanhaesebroeck *et al.,* 2010). Complete activation of AKT depends on the phosphorylation of two key residues: threonine residue 308 (Thr308), which leads to the activation of the catalytic protein kinase core, and serine residue 473 (Ser473) in a C-terminal hydrophobic motif, which stabilizes Thr308 phosphorylation. Once activated, AKT interacts with many targets, regulating multiple signaling pathways (Manning & Toker, 2017) and directly phosphorylates protein targets, transcription factors, metabolic enzymes, and can promote cell survival, proliferation and changes in cellular metabolism through various downstream targets.

At the oocyte maturation level, AKT activity has been related to the resumption of meiosis, the formation of the meiotic spindle and the expulsion of the second polar body in M-II (Cecconi

et al., 2010). The primordial to primary follicle transition is controlled through the induction of PI3K/AKT pathway, which is expressed primarily by granulosa cells present in oocytes and PI3K recruitment from the cytoplasm to the cell membrane through the binding of the SH2 domain (Cecconi *et al.,* 2010; Liu *et al.,* 2007). Moreover, Kimura et al. (2008), confirmed the crucial role of AKT in promoting survival and proliferation of the primordial germ cells and found that proliferation increased upon AKT induction. Phosphorylated AKT controls the activity of the serine-threonine glycogen synthase kinase 3 (GSK3) and the FOXO family members (FOXO1, FOXO3a and FOXO4). In the bovine ovary, GSK3 is localized in the cytoplasm of granulosa cells and oocytes (Cecconi *et al.,* 2012). In the mammalian ovary, FOXO3a controls follicular activation and early development and it has been detected in the nucleus of oocytes (Liu *et al.,* 2007). On the other hand, intra-oocyte FOXO3a inhibits the production of BMP-15 and may also suppress the activation of the Smad pathway that controls the proliferation and differentiation of surrounding granulosa cells. The PI3K/AKT pathway is subjected to TGF-β/BMP modulation that is involved in the regulation of granulosa cell proliferation and follicle survival/apoptosis (Cecconi *et al.,* 2012). This suggest a link between BMP signaling, PI3K/AKT and RAS/ERK pathways in the regulation of key cellular processes involved in cell growth and proliferation (Manning & Toker, 2017).

Although PI3K is an upstream activator of AKT, also phosphorylates other target proteins containing pleckstrin homology domains such as PDK1 and PLCγ to mediate crucial cellular processes, including cell growth or gene transcription (Manning & Toker, 2017). During embryonic development PI3K/AKT is a major signaling pathway to coordinates processes necessary to maintain survival and its inhibition can cause a significant delay in blastocyst hatching (Riley *et al.,* 2005; Zhang *et al.,* 2007). The developing embryo is dependent on signals generated by growth factors produced by the embryo itself or present in the maternal environment and expresses several growth factor receptors known to activate the PI3K pathway (Baran *et al.,* 2013). Following fertilization, AKT is expressed from 1-cell stage to morula on cell surface (Baran *et al.,* 2013; Cecconi *et al.,* 2012), while at blastocyst stage is present in cells of the TE.

Chen et al. (2016), in a study of mouse preimplantation embryogenesis showed that AKT was permanently located in the cytoplasm from 1-cell stage to early 4-cell stage embryos and this localization was generally consistent with minor and major EGA, which strongly implied that AKT and their phosphorylated form (p-Ser473) may get involved in the EGA and could interact with the cell cycle regulatory proteins. However, further research is required for better understanding the interaction of AKT and their phosphorylated forms into the mammal preimplantation embryogenesis.

1.3. Early embryo development *in vitro*

In vitro fertilization (IVF) and embryo culture try to simulate the conditions that occur *in vivo* to provide high quality embryos capable of continue development and implantation and allows us to enhance our understanding of early embryo development, above all during the preimplantational stages. Therefore, IVP of embryos is divided in three steps: *in vitro* maturation (IVM), IVF and *in vitro* culture (IVC). Furthermore, considering the many similarities existing between bovine and human embryonic development, bovine IVP is suggested as one possible model for human reproduction (Ménézo & Hérubel, 2002).

1.3.1 *In vitro* maturation

Oocyte maturation is a required physiological event for successful fertilization and embryo development, and its quality is the main factor determining blastocyst yield. Oocyte developmental competence is associated with the size of the antral follicle from which it is recovered and the stage of the follicular wave. IVM is initiated immediately following the removal of immature oocytes from small to medium-size antral follicles although, it has been demonstrated that oocytes recovered from follicles of more than 6 mm yield a higher proportion of blastocysts compared with follicles between 2-6 mm (65.3% and 34.3% respectively) (Lonergan *et al.,* 1994). Obtained cumulus–oocyte complexes (COCs) then are selected for IVM by morphological criteria based on the appearance of the cytoplasm and the presence or absence of CCs.

The CCs surrounding the immature oocyte play a critical role on further development as they are channeling metabolites and nutrients to the oocyte to stimulate GVBD and drive development to M-II (Assidi *et al.,* 2010; Lonergan & Fair, 2016). This entails the action of numerous metabolic pathways that govern certain aspects of CCs metabolism (Downs, 2015), such as their expansion and gene expression, characteristics considered as morphological indicators of the successful completion of oocyte maturation as well as marks of oocyte quality (Bunel *et al.,* 2015). According to these features the COCs are classified into 4 groups (Figure 6) (Loos *et al.,* 1989):

- Grade 1: oocytes with more than 4-5 layers of CCs, complete and compact, and a homogeneous cytoplasm.
- Grade 2: oocytes with fewer layers of CCs, between 1 and 3, a homogeneous cytoplasm or a darker area on the perimeter of the oocyte.
- Grade 3: oocytes without CCs, with darker and/or irregular cytoplasm.
- Grade 4: expanded CCs, and pyknotic or very dark and/or irregular oocyte cytoplasm.

After obtaining and selecting the COCs from the pre-antral follicles by their morphology, IVM is performed by placing them in a suitable culture medium for 24 h in 38.5 ºC, 5% of CO_2 in air and maximum humidity to support the spontaneous resumption of meiosis, nuclear and cytoplasmic maturation and development of the oocyte to M-II (Rizos *et al.,* 2002a).

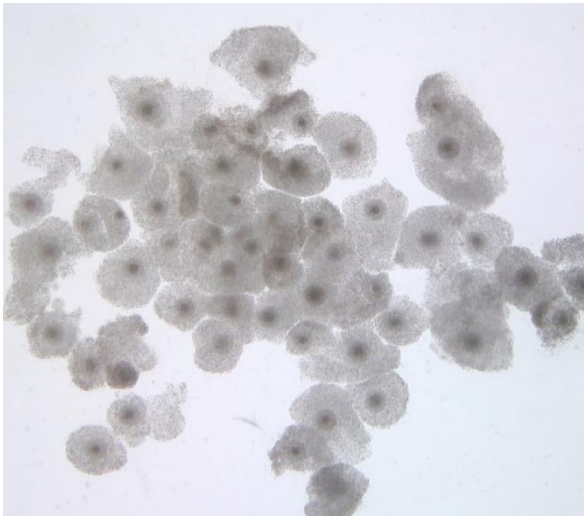

Figure 6. COCs grade 1 and 2.

1.3.1.1. Nuclear maturation

Nuclear maturation refers to the transition from GV nucleus to a second metaphase arrangement of the chromosomes and formation of a first polar body. This process is controlled by three key biochemical pathways: (1) MPF, which activates meiotic resumption resulting in GVBD; (2) anaphase promoting complex, which regulates progression from GVBD; and (3) cytostatic factor, which maintains meiotic arrest at M-II (Fair, 2013). Regulation of MPF is via the activity of several kinases and phosphates, whereas anaphase promoting complex and cytostatic factor are modulated mainly by translational regulation through sequential waves of polyadenylation and deadenylation (Belloc *et al.,* 2008).

In vivo resumption of meiosis in the oocyte within the dominant follicle is related to the preovulatory surge of LH, which triggers GVBD and progression to M-II (Picton *et al.,* 1998; Sirard *et al.,* 1998; Smith, 2001). In the case of IVM, spontaneous resumption of meiosis occurs before the completion of cytoplasmic maturation, compromising developmental competence and this is because oocytes aspirated from the ovary with the dominant follicle just before LH surge display alterations in their nuclear and cytoplasmic morphology (Lonergan & Fair, 2016).

On the other hand, by delaying spontaneous resumption of meiosis *in vitro* using a meiotic inhibitor, it has been hypothesized that continued mRNA and/or protein accumulation in the oocyte might enhance the maturation process. Although it is possible to reversibly inhibit meiotic

resumption, evidence for a positive effect on oocyte competence is relatively sparse, but studies using pharmacological compounds that allow synchronization of maturation processes and that prolong interaction between the immature oocyte with CCs have reported promising results (Lonergan & Fair, 2016).

Nevertheless, nuclear maturation alone is not sufficient to produce a high-quality oocyte. A recent metanalysis of various models of M-II –stage oocytes leds to the identification of 56 candidate genes associated with oocyte quality across several species, as well as Wnt signaling and PI3K/AKT pathways being crucial for the maturation of high quality oocytes (Fair, 2013; O'Shea *et al.,* 2012).

1.3.1.2 Cytoplasmic maturation

Oocyte cytoplasmic maturation is characterized by a series of ultrastructural and molecular changes. In immature oocytes the high metabolic activity is reflected by a larger number of organelles in the ooplasm such as mitochondrial clusters and lipid vacuoles, but during oocyte IVM, the synchronization of nuclear and cytoplasmic maturation is of fundamental importance ensure the progression of early embryo development (Ferreira *et al.,* 2009). The cytoplasmic maturation includes relocation and modification of organelles, acquisition of functional Ca^{2+} release mechanisms and capacity to decondense the chromatin of the fertilizing sperm among others (Ferreira *et al.,* 2009).

Organelles modification during cytoplasmic maturation: Organization and continued metabolic activity of mitochondria are necessary features for cytoplasmic maturation and resumption of meiosis, affecting subsequent development after fertilization (Ferreira *et al.,* 2009). The preimplantation period of development is sustained by mitochondria produced during oogenesis, and only when the embryo begins implantation, their production is resumed (Ferreira *et al.,* 2009).

a) *Mitochondrial distribution and metabolic activity:* In bovine oocytes, the major relocation of mitochondria occurs during IVM and is influenced by hormones and energy substrates of the maturation medium (Eichenlaub-Ritter *et al.,* 2011; Ferreira *et al.,* 2009). Confocal studies revealed a higher incidence of mitochondrial clustering in the cytoplasmic periphery of oocytes matured *in vitro* in standard tissue culture medium 199 (TCM-199) containing glucose and lactate and supplemented with serum, whereas mitochondria of oocytes matured in chemically defined poor medium, often appear homogeneously distributed (Ferreira *et al.,* 2009; Krisher & Bavister, 1998). The energy status (ATP content) of oocytes is critical for their maturation and has been suggested as an indicator

for the developmental potential (Eichenlaub-Ritter *et al.,* 2011). Embryos with less ATP in the cytoplasm had slower development and resulted in a smaller number of cells (Stojkovic *et al.,* 2001). Moreover, the mitochondria are involved in ATP synthesis, reactive oxygen species (ROS) production and apoptosis (Eichenlaub-Ritter *et al.,* 2011). Mitochondrial dysfunctions or abnormalities may compromise developmental processes by inducing chromosomal segregation disorders, maturation and fertilization failures, or oocyte/embryo fragmentation resulting in mitochondria-driven apoptosis.

b) *Cortical granules and distribution:* CGs are small vesicles that contain enzymes. During meiosis resumption, the CGs migrate from the Golgi apparatus to the vitelline surface, assuming a position 0.4–0.6 μm below the plasma membrane, and such distribution contributes to block polyspermia (Hosoe & Shioya, 1997). If fertilization with more than one spermatozoon occurs, the resulting zygote will undergo abnormal cleavage and will become non-viable, eventually degenerating at the beginning of mitotic divisions (Ferreira *et al.,* 2009). Dispersion of CGs is regarded as being essential for the proper course of oocyte maturation. Improvements of maturation culture conditions would enhance migration of CGs in oocytes and will contribute to embryo development up to the blastocyst stage.

1.3.1.3. Molecular maturation

Molecular maturation corresponds to the phases of oocyte growth and maturation. It involves the transcription, storage and processing of the mRNAs which are stored in a stable, inactive form until translational recruitment and are required for maturation, fertilization and early embryogenesis (Ferreira *et al.,* 2009). It has been hypothesized that the quality of an oocyte is based on the presence of the appropriate set of mRNAs and proteins stored into ribonucleoprotein particles (Wrenzycki *et al.,* 2004). This correct storage is of great importance since many transcripts will be consumed until the EGA. It is known that several mechanisms are involved in the activation of translationally inactive mRNAs (Tomek *et al.,* 2002), such as polyadenylation. In a study on the polyadenylation of various mRNAs involved in the oocyte meiosis resumption to the first embryo cleavages the authors observed several differences in the polyadenylation patterns and concluded that these specific changes in mRNA polyadenylation are associated with modulation of gene expression in the embryos (Ferreira *et al.,* 2009) and abnormal levels of polyadenylation in some maternal mRNAs are accompanied by a lower potential for embryo development.

In bovine COCs, the polyadenylation of mRNAs code for the proteins necessary for MPF and MAPK activation is important, thus, appropriate synthesis of these transcripts in the oocyte cytoplasm will also be crucial for the nuclear resumption and progression of meiosis (Filatov *et al.,* 2019). In addition to cell cycle regulators, other critical molecules for the development and early embryogenesis are synthesized and accumulated inside the oocyte, with their presence considered to be a marker of having acquired cytoplasmic competence to support the subsequent phases (Ferreira *et al.,* 2009; Krischek & Meinecke, 2002). Eichenlaub-Ritter et al. (2011) showed that a deficiency in oocytes gene expression that occur during follicle development might be related to an altered composition of the same. Gene expression profiling of *in vivo*- and *in vitro*-matured bovine oocytes can identify transcripts related to their developmental potential (Camargo *et al.,* 2019). Some of the most important genes are the glucose transporter (*GLUT1*) (Augustin *et al.,* 2001), glucose-6-phosphate dehydrogenase (*G6PD*) and those genes associated with oxidative response: manganese superoxide dismutase (*MnSOD*) related to mitochondrial activity and glutathione peroxidase (*GPX1*) (Guérin *et al.,* 2001). Within this context, glutathione is one of the markers of cytoplasm maturation that has been extensively investigated, this enzyme plays a fundamental role in cell protection against oxidative damage (Ferreira *et al.,* 2009) by eliminating the ROS produced during mitochondrial metabolism. Investigation of the oocyte maturation mechanisms is one of the top priorities of contemporary developmental biology. However, as highlighted by Filatov et al. (2019) the mechanisms of meiotic resumption of oocytes are extremely complex and there are certain mechanisms that require further investigation such as the role of the MAPK cascade and many other signaling molecules that regulate the initiation of meiotic resumption.

1.3.2 *In vitro* fertilization

IVF consists of the co-incubation of matured oocytes and capacitated sperm under the right conditions in order to produce a viable zygote. The selection of motile sperm is a prerequisite for successful IVF. There are several products and protocols to do so: the swim-up, and gradients such as Percoll and BoviPure™ (Samardzija *et al.,* 2006). Both gradients have similar results regarding the final rate of blastocysts, but the advantage of BoviPure™ is that it is less toxic (Samardzija *et al.,* 2006). After selection, the sperm concentration is adjusted normally to 10^6 sperm/mL and coincubated with the matured oocytes normally for 18 h, at 38.5 ºC, 5% CO_2 in air and saturated humidity. During this period spermatozoa cross all physiological barriers, fuse with the ooplasm, activate the oocyte, and then pronuclear formation begins (Galli *et al.,* 2003).

The media used are designed with a specific ionic balance for sperm requirements and contain heparin that capacitates the sperm and prepares it for the acrosome reaction (Parrish, 2014). The most commonly used medium is the TALP, a modification of the Tyrode´s medium that contains sodium bicarbonate, albumin as a source of protein and lactate and pyruvate as an energy source (Gordon, 2003). The presumptive zygotes obtained after IVF are selected based on their morphological characteristics like homogeneity of the ZP, perivitelline space and cytoplasm and placed into culture.

1.3.3. *In vitro* culture

IVC consists of the culture from the presumptive zygotes (Day 1 after IVF) up to the blastocyst stage (Days 7-9). The relevance of IVC resides in the fact that postfertilization culture conditions can dramatically alter the quality of the embryo (Rizos *et al.,* 2002). The factors that most influence the quality of the embryos are the conditions after fertilization; culture medium, number of embryos in culture, temperature and gas balance in the incubator (Lonergan, 2007). Over the years, different embryo culture systems in defined or non-defined media (in which the concentration of the major components is unknown) with supplementation of fluids from the reproductive tract (oviductal and uterine fluids), or their extracellular vesicles (EVs) or co-culture with oviduct epithelial cells have been described (Lopera-Vasquez *et al.,* 2017; Rizos *et al.,* 2017). The most used medium for embryo culture is synthetic oviductal fluid (SOF) which is usually supplemented with 5% of foetal calf serum (FCS) and/or bovine serum albumin (BSA) (Holm *et al.,* 1999; Tervit *et al.,* 1972). However, it has been shown that serum negatively affects embryo quality compared to the production with albumin in a concentration of 3 mg/mL (Rizos *et al.,* 2003). The addition of FCS to the culture media has also been linked to the large offspring syndrome, which is characterized by large calves that have a high postnatal mortality due to defects in many organs (Young *et al.,* 1998).

Overall, the goal of *in vitro* embryo production is to simulate as closely as possible the conditions that occur *in vivo*, to obtain high quality embryos capable of resulting in viable births. However, despite the success in the development and use of these technologies, IVP is an inefficient (the proportion of embryos reaching the blastocyst stage is around 40%) and suboptimal process (embryos that do reach the blastocyst stage are often compromised in quality and competence) (Lonergan & Fair, 2008). Improvements in IVP has been evidenced by "mimicking" physiological embryo culture conditions such as: co-culture with somatic cells monolayers mainly from the reproductive tract (granulosa or oviductal epithelial); oviductal and uterine fluids; and their EVs (Hamdi *et al.,* 2018; Lopera-Vasquez *et al.,* 2017). However, obtaining

good quality embryos, competent for establishing a pregnancy after transfer to a recipient remains a challenge for further research.

1.3.3.1. *In vitro* conditions affecting embryo development and quality

Embryo culture is the longest step of the IVP embryo process and it is when greatest reduction in development occurs (30 - 40% of blastocysts rate) (Rizos *et al.,* 2008).

It has been shown that the intrinsic quality of the oocyte is the main factor affecting blastocyst yield since could influence the transcriptome of the matured oocyte while the postfertilization culture environment impacts the quality of the produced blastocysts (Sirard, 2012). To highlight the importance of *in vitro* systems, different experiments have been carried out alternating *in vitro* with *in vivo* embryo culture, where blastocysts produced from embryos maintained for different time intervals in *in vivo* culture had different survival rates (Rizos *et al.,* 2002; Tesfaye *et al.,* 2007).

It has been demonstrated that the dynamic of embryo development and embryo quality can be affected by suboptimal *in vitro* conditions manifested by a darker morphology (Rizos *et al.,* 2002a), lower cryotolerance (Rizos *et al.,* 2008), altered ICM/TE ratio (Salzano *et al.,* 2014) and altered gene expression patterns (Niemann & Wrenzycki, 2000) when compared to their *in vivo* counterparts. These results led to consider the fact that the period around the time of EGA is critical for the quality of the embryo. Gad et al. (2012) investigated the consequences of culture conditions before and during the EGA on bovine embryonic developmental rates and global gene expression patterns. Embryo development was similar irrespective of where culture took place; however, the transcriptome of the blastocysts was clearly influenced by culture conditions, confirming once again the significant role of culture conditions during EGA (Gad *et al.,* 2012; Graf *et al.,* 2014).

During IVC, compaction and cavitation of the morula are crucial processes in early embryo development. It has been shown that the addition of FCS to the culture medium decreases the degree of compaction of the blastomeres in the morula stage and they present a greater accumulation of lipid droplets in their cytoplasm (Rizos *et al.,* 2003), parameters that affect embryo cryotolerance. Compaction is a prerequisite for TE formation, involving cell adhesions between blastomeres mediated by E-cadherin and tight junction proteins (Van Soom *et al.,* 1997). The blastocoel formation is accompanied by the formation of TE and the ICM (Watson & Barcroft, 2001). Compaction and cavitation are dependent on adequate embryonic gene transcription. Moreover, *in vitro* manipulation of embryos has also been related to oxidative stress generation and the production of substances such as ammonia or oxygen radicals that can produce lipid

peroxidation, membrane injury and structural damage, leading to decreased cryotolerance, apoptosis and alterations in gene expression compared to *in vivo* produced embryos (Moore *et al.,* 2007; Somfai *et al.,* 2007).

During the period of epigenetic reprogramming embryo is especially vulnerable to *in vitro*-induced epigenetic defects (El Hajj & Haaf, 2013), these alterations of the epigenetic profile may have a direct effect on the subsequent embryo and fetal development. Also, a correctly programmed oocyte can support the subsequent embryo development, evidenced in the efficiency and quality of blastocysts produced *in vivo* (Graf *et al.,* 2014). *In vitro* embryo culture has been associated with abnormal reprogramming of imprinted genes involved in blastocyst formation such as cell-to-cell adhesion (E-cadherin, connexins and tight junctions genes), cell communication (gap junctions), differentiation marks and genes related to metabolism, growth, and differentiation (Miller *et al.,* 2003). In some cases, the significant increase in the expression of those genes supports the hypothesis that current *in vitro* culture systems are associated with a considerable amount of oxidative stress, which can alter the normal development of the embryo (Bennemann *et al.,* 2018).

1.3.3.2. Evaluation of embryo quality.

The best method to assess the quality of each embryo produced is its ability to establish and maintain a pregnancy after transfer to a recipient. However, for practical and economic reasons, it is only ever possible to transfer a subset of embryos. Therefore, alternative methods for evaluating embryo quality have been developed and are considered valuable tools to understand or identify the *in vitro* culture conditions.

a) Morphology

The blastocyst morphology is the first parameter analyzed for immediate transfer or cryopreservation. Under light microscope, *in vivo* embryos present a lighter color when compared to their *in vitro* counterparts (Fair *et al.,* 2001; Rizos *et al.,* 2002a). Furthermore, Fair et al. (2001), elucidated the effect of culture environment on blastocyst's morphology demonstrating that blastocysts cultured in SOF with FCS had wick cell-to-cell contacts compared to the *in vivo* produced. Another study evidenced that *in vivo* embryos present a higher plasma membrane attachment to ZP, a dense-continuous cover of microvilli and a small number of lipid droplets, while the *in vitro* counterparts exhibited less and discontinuous microvilli over the plasma membrane, and increased lipid content (Figure 7) (Rizos *et al.,* 2002a). It is well documented that higher lipid content in blastocyst is related to a lower cryotolerance (Abe *et al.,* 2002). Hence, the differences observed at ultrastructural level between *in vivo* and *in vitro* embryos, even between

embryos produced *in vitro* in different culture systems, may in part explain the cryotolerance variations observed.

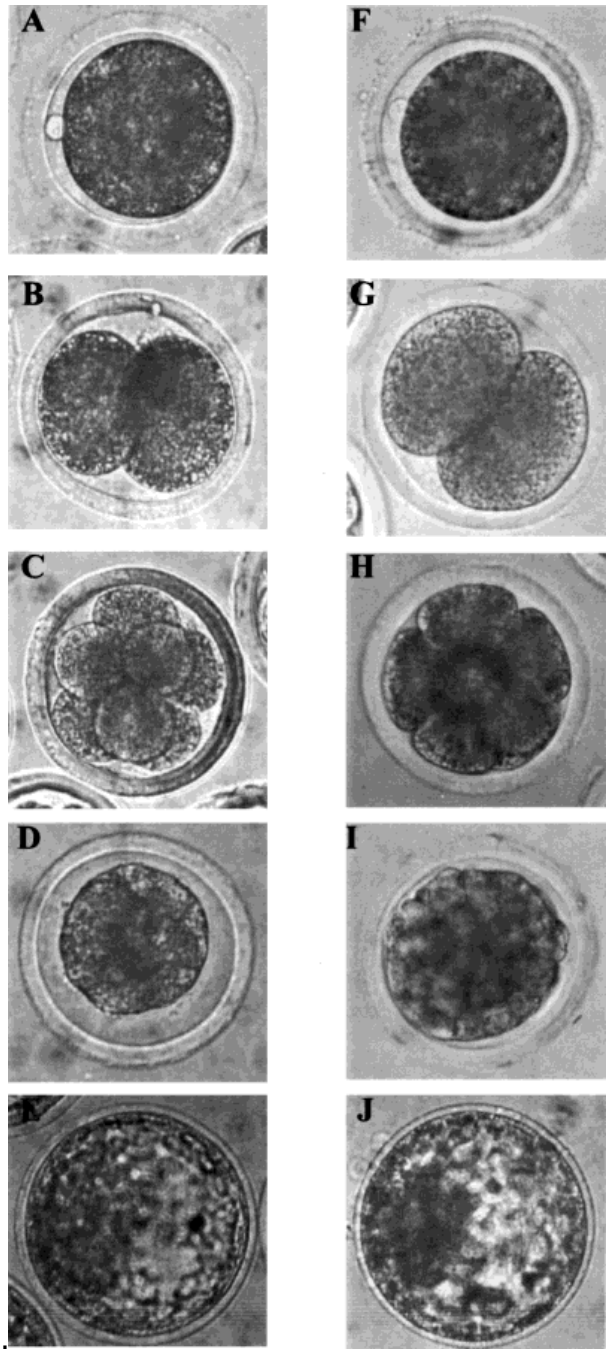

Figure 7. Morphology of bovine embryos produced *in vivo* (A±E) or *in vitro* (F±J). Images are representative of matured oocytes (A and F), 2-cell embryos (B and G), 8-cell embryos (C and H), morula (D and I), and blastocysts (E and J) (Rizos *et al.,* 2002a)

b) Embryo cell number

Differential staining can be used to obtain a better estimation of embryo quality and differentiation. An asynchrony in the mitotic division of the blastomeres could be reflected in the

compaction stage of the morula and in the subsequent ICM and TE cells differentiation (Johnson & Ziomek, 1981). Therefore, this technique allows to compare ICM and TE development in different culture conditions to evidence deviations in embryo development (Van Soom *et al.,* 2001). Clemente et al. (2009) used differential cell count to evaluate the quality of *in vitro* produced bovine blastocysts in presence or absence of progesterone. Similarly, Rodríguez et al. (2006) demonstrated that retinoic acid and its specific receptor agonist increase the number of cells in the ICM and TE of *in vitro* produced bovine embryos and Trigal et al. (2011) evaluated the use Activin A under *in vitro* conditions on bovine embryo development and quality, finding a lower number of TE cells in embryos cultured in absence of Activin. Recently, Chowdhury et al. (2017) evaluated that supplementation of lycopene during oocyte maturation or L-ergothioneine during embryo culture (Zullo *et al.,* 2016a) increase the quantity of TE and ICM cells, confirming a beneficial effect on embryo quality. Therefore, supplementation of the medium with different substances can have a positive effect on embryonic development by influencing the kinetics of cell division.

c) Gene Expression

Early developmental stages include the timing and progression through the first and subsequent cellular divisions, formation of necessary gap junctions, EGA, compaction, differentiation of cell types, and secretion of embryonic gene products essential for maternal recognition. Transcriptomics can serve as an excellent tool to assess the roles of key genes and pathways involved in the different stages of embryo development (Khatib & Gross, 2019).

Kues et al. (2008) studied the volume of embryonic mRNA transcripts through the different stages of development and concluded that the observed lower number of 4-to 8-cell stage transcripts was due to rapid degradation of maternal transcripts during maternal embryonic transition. Moreover, 350 genes were developmental stage–specific, with about 70 genes highly expressed in the 2-cell stage that were then downregulated in subsequent stages, demonstrating high metabolic and signaling activity, thus providing evidence of the dynamic changes that occur within the embryo that lead to EGA (Kues *et al.,* 2008). In addition, analysis of expression patterns of important genes in early development provides valuable information about the effect of environmental factors on the early embryo. The products of these genes are involved in various biological processes including metabolism, growth factors, cytokine signaling, stress adaptation, transcription and translation, epigenetic regulation of transcription, apoptosis, compaction and blastocyst formation (Wrenzycki *et al.,* 2004).

It is well documented that gene expression is different in IVP oocytes and embryos compared to their *in vivo* counterparts. The expression of DNA Methyltransferase 1, 3A, and 3B

(DNMT1, DNMT3A and *DNMT3B)* has been demonstrated to be upregulated in bovine oocytes after IVM compared to *in vivo*-matured oocytes (Heinzmann *et al.,* 2011). Furthermore, it has been reported that between different culture media, genes related to apoptosis and oxidative stress (*BAX*, *SOX*, *HSP70*) had different expression patterns (Sagirkaya *et al.,* 2006). When comparing *in vivo*-derived and IVP bovine embryos, genes related to metabolism, growth, and differentiation (Glucose transporter type 5 (*GLUT-5)*, Conexin 43 or gap junction protein alpha1(*CX43)*, Insulin like growth factor 2 (*IGF-II);* Leukemia inhibitory factor (*LIF)*) were upregulated in embryos derived *in vivo*, while genes related to stress (*SOX*, *MnSOD*, *BAX*, *HSP70.1*, *PRDX5*) were upregulated in IVP embryos. The significant increase in expression of those genes supports the hypothesis that current *in vitro* culture systems are associated with a considerable amount of oxidative stress (Rizos *et al.,* 2003; Wrenzycki, 2018).

d) Artificial intelligence as a new tool to address the quality of mammalian embryos

Currently, the possibility of using an artificial intelligence system is limited only to the morphological analysis of the embryo, nevertheless theoretically, it is possible to adapt the system for the direct prediction of successful embryo implantation, once the variables that describe the physiological, endocrinological and metabolic environments of the recipient are included on the machine learning algorithms (Rocha *et al.,* 2016). However, in light of the multiple attempts to develop a precise non-invasive system for embryo classification, this is still an ongoing process. Recently, the use of mathematical and statistical tools for the analysis of embryo viability has been proposed. Among the main researches, van Loendersloot et al. (2014) reported the use of multivariate logistic regression for the classification of embryos according to implantation potential, being able to categorize embryos with high, moderate or low potential. In the same line, Chen et al. (2016a) proposed the use of a computer-assisted scoring system to have a higher discriminatory power for embryo selection, together with a multivariate logistic regression system, and multivariate adaptive regression splines. The most promising alternatives seems to be the ones that take into account the metabolites used by the embryo and obtained by analysis of the conditioned culture medium, the use of applied mathematics and statistics with the classificatory system or dedicated software for the analysis of kinetics, symmetry or morphology of the embryo.

1.4. The physiological environment of early embryo

The gaseous environment to which the oocyte is exposed varies considerably between *in vitro* and *in vivo* conditions. The female reproductive tract is rich in antioxidants and is

considerably different from the synthetic medium used for IVC of oocytes and embryos (Wrenzycki & Stinshoff, 2013).

When oocytes and embryos are cultured *in vitro*, they are exposed to manipulation, light, temperature and O_2 concentration, which favor the increase of ROS, which in a balanced way can act as signaling molecules in different physiological processes (Guérin *et al.,* 2001; Yu *et al.,* 2014). Therefore, studies regarding the components of the media such as metabolites, nutrients, gaseous atmosphere or use of substances with antioxidant properties during IVP are essential to avoid excessive ROS increase, lipid droplet formation and to improve the quality of produced embryos (de Matos *et al.,* 2002; Kere *et al.,* 2012).

The parameters that influence the physiological environment are:

1. **Culture medium:** The success of IVM suffers from differences between the composition of the *in vitro* culture media compared with *in vivo* conditions (Sutton *et al.,* 2003); hence, an understanding of intrafollicular conditions is critical for successful oocyte selection and maturation regardless of species. Follicular fluid contains a variety of proteins, cytokine/growth factors, other peptide hormones, steroids, energy metabolites and other undefined factors (Leroy *et al.,* 2011; Sutton *et al.,* 2003; Van Hoeck *et al.,* 2013). The presence and growth of the follicle is likely to affect nutrient levels reaching the oocyte, and intrafollicular conditions may have an influence on the developmental competence of the oocytes. Some of the follicular fluid/IVM media components are involved in a variety of processes and pathways affecting the developmental competence of the COCs (Wrenzycki & Stinshoff, 2013). The media used for IVM are known to affect both maturation and subsequent embryonic development and they can be classified as simple or complex media. Simple media are buffered salt solutions with energy sources such as pyruvate, lactate and glucose, whereas complex media contain in addition a mixture of amino acids, vitamins, and other molecules (Gordon, 2003). In the bovine oocyte maturation, TCM-199 is the complex medium more extendedly used and is supplemented with macromolecules such as FCS or BSA, gonadotropins, steroids, antioxidants and growth factors (Gordon, 2003; Lonergan & Fair, 2008). Further refinement of conditions for IVP in order to improve oocyte and embryo quality included addition of biologically active agents (e.g., FSH and various growth factors) involved in the signaling between the developing oocyte and the surrounding GCs that have been identified (Machaty *et al.,* 2012). Most recent experimental data indicate that EGF-like growth factors accumulate in the follicle at ovulation and are potent stimulators of oocyte maturation and cumulus

expansion (Hsieh *et al.,* 2009). The incorporation of such factors in the maturation medium may improve the quality of matured oocytes.

In the last years, in an attempt to mimic *in vivo* conditions and improve the quality of the embryos produced, different systems of embryo culture have been developed such as co-culture *in vitro* with bovine oviductal epithelial cells (Lopera-Vásquez *et al.,* 2016; Ulbrich *et al.,* 2010), the use of oviductal fluid (Lopera-Vasquez *et al.,* 2017) or use of EVs as a supplement during *in vitro* embryo culture. Thus, the presence of EVs in oviductal fluid and their effect on early embryonic development may be of great importance in early embryo-maternal communication and improve embryo quality in our current IVP systems.

Another factor considered during embryonic development is oxidative stress, which is why it has been shown that supplementation of the culture media with antioxidants helps to maintain intra- and extracellular redox balance, which is necessary to reduce the toxicity of ROS, improving embryo development, increasing cryotolerance and cell differentiation, and inhibiting apoptosis during culture (Takahashi, 2012). Vitamins such as tocopherol, ascorbic acid or folic acid play a key role in reducing oxidative damage and improving blastocyst development rate in mouse embryos (Wang *et al.,* 2002). Also, the supplementation of culture media with antioxidants, such as crocetin or retinol, seems to have a positive effect on development, embryo quality and improvement of the cryotolerance of bovine embryos (Zullo *et al.,* 2016b).

2. **Energy sources:** Changes in the energy needs of COCs during maturation have been investigated and addressed based on the different requirements of the oocyte and CCs.
   - Glucose: The capacity of the oocyte to utilize glucose may be used as a predictive marker of oocyte quality (Krisher & Bavister, 1998), since its addition to the medium improves the resumption of meiosis, embryo cleavage, morula and blastocyst rates in cattle (Krisher & Bavister, 1998). However, high concentrations of glucose during IVM can lead to an increased generation of ROS and decreased intracellular glutathione (GSH) availability (Hashimoto *et al.,* 2000). On the same line, Perkel et al. (2015) reported that the transition from the morula to the blastocyst stage and the ability to metabolize glucose appears to be a sign of an embryo's growth and potential and higher glucose uptake has also been correlated with viability (Gardner *et al.,* 2011). With all these precedents, glucose supplementation to culture media would provide an improvement to the IVP conditions.

- Pyruvate: Downs et al. (2002) reported that pyruvate consumption correlates with meiotic maturation. Moreover, metabolomic studies with meiotically active oocytes have highlighted the importance of pyruvate in culture as a potential biomarker to assess early embryonic viability (Perkel *et al.,* 2015). Rieger et al. (1992) reported that bovine embryos that developed to the blastocyst stage used more pyruvate and lactate in early preimplantational development. A second function of pyruvate is to protect the embryo against oxidative stress (Morales *et al.,* 1999) since pyruvate can produce water, carbon dioxide and acetate, suggesting that higher production of acetate by embryos can be a sign of oxidative stress and decreased viability. Therefore, pyruvate uptake alone may be a major predictor of embryo development and viability due to alternative interpretations of its use by the embryo; however, this metric could be very powerful if used in combination with other metabolites such as glucose or lactate (Perkel *et al.,* 2015). On the other hand, lactate production increases significantly throughout early preimplantation development. Compaction, expansion, and blastocoel formation require large amounts of energy, which is produced through glycolysis (Khurana & Niemann, 2000) and a high IVP of lactate is thought to be a consequence of metabolic stress associated with the unnatural culture environment. Nevertheless, the consistent rise in lactate production throughout development of the preimplantation bovine embryo could be a potential indicator of viability (Perkel *et al.,* 2015).

3. **Oxygen concentration (and reactive oxygen species):** Oxygen consumption is a potential quality parameter as it provides an indication of the overall metabolic activity of a single embryo (Harvey, 2007). The correlation between oxygen consumption and morphological quality, is the main parameter used in embryo evaluation (Lopes *et al.,* 2007). Physiologically, the oxygen concentration in follicular fluid and in the oviduct of mammals varies between 2 and 13% (Harvey, 2007). Hashimoto (2009) observed that a reduced oxygen concentration (5%) and supplementation of the maturation medium with glucose led to increased blastocyst rates indicating a close connection between glucose and oxygen metabolism. On the other hand, studies demonstrated that very high levels of O_2 (20%) can result in a greater presence of ROS, which can be harmful to the oocyte (Cetica *et al.,* 2001; Guérin *et al.,* 2001; Hashimoto, 2009). Studies in mice concluded that 5% oxygen was optimal for nuclear maturation and that values below 5% were considered detrimental to maturation. After fertilization, blastocysts derived from mouse oocytes matured at 5% oxygen had significantly higher cell numbers and reduced metabolism, as measured by glucose uptake and lactate production compared to those matured at 20% (Preis *et al.,* 2007).

Mingoti et al. (2011) found a higher development when embryos were cultured with 20% O_2 and the medium was supplemented with serum, that was confirmed as well with antioxidant agents supplementation (Rocha-Frigoni *et al.,* 2015). In addition, it has been demonstrated that combination of oxygen tension and serum supplementation plays a major role in the lipid profile of bovine embryos (Ferreira *et al.,* 2010). On the other hand, another study indicated that different O_2 levels (5% or 20%) during IVC embryo do not affect embryonic development but had a consequence on the epigenetic process, such as histone marks histone 3 lysine 9 dimethylation (*H3K9me2*) in bovine blastocysts (Gaspar *et al.,* 2015). Furthermore, Bennemann *et al.,* (2018) reported that energy substrates required for oocyte maturation may differ under low and high oxygen tensions. During nuclear and cytoplasmic maturation the oocyte is susceptible to glucose concentration-dependent perturbations and most of the ATP is provided by glycolysis or imported from GCs (Harvey, 2007). However, the indicators of an altered metabolism in both oocyte and embryo are the mitochondria, moreover, their distribution is important to maintain energy homeostasis. An altered distribution pattern due to IVM conditions may be an indicator of stress and decreased developmental potential (Banwell & Thompson, 2008). In addition, the mitochondrial membrane potential has been shown to be higher in *in vivo* matured oocytes compared to those matured *in vitro* with 20% oxygen (Preis *et al.,* 2007).

Besides, high oxygen tension is associated with higher levels of ROS and may exert harmful effects on the development of bovine embryos and on cellular function by inducing oxidative damage of intracellular components (Guérin *et al.,* 2001; Yuan *et al.,* 2003). ROS are powerful oxidants physiologically produced during activation of gamete and embryo metabolism (Agarwal *et al.,* 2008) and oxidative stress occurs when the ROS production level exceeds the capacities of the cellular antioxidant defense systems, resulting in cellular injuries, such as lipid peroxidation, mitochondrial damage, amino acid and nucleic acid oxidation and apoptosis (Rocha-Frigoni *et al.,* 2016). At low concentrations, ROS play important physiological roles *in vitro* such as regulating the rate of pre-implantation embryo development (Combelles *et al.,* 2009). The balance between ROS and antioxidants may have a critical role in reproductive processes such as folliculogenesis as documented by the presence of various antioxidant enzymes within the follicular fluid (Gupta *et al.,* 2011). However, antioxidant supplementation *in vitro* must be balanced with oxygen concentration, thus the addition of antioxidants molecules alone may not be enough to combat the damaging effects of ROS (Guérin *et al.,* 2001).

4. **Antioxidants:** The use of substances with antioxidant properties during IVP of embryos may avoid the excessive increase of ROS, protect the embryo against oxidative stress and improve the production by decreasing ROS levels directly or indirectly by increasing GSH levels in oocytes (Torres *et al.,* 2019; Wang *et al.,* 2014). IVP media show high ROS concentrations, which make it necessary to add antioxidants. A wide variety of antioxidants have been used to reduce ROS during IVP of embryos (Mishra *et al.,* 2016; Rocha-Frigoni *et al.,* 2016).

Exogenous antioxidants play a key role in the delicate equilibrium between oxidation and antioxidation and antioxidant activities intimately depend on their concentrations. In most circumstances, physiological doses of antioxidants exert beneficial effects; while overdoses of them exhibit detrimental effects (Bouayed & Bohn, 2010). Intracellular antioxidants (cysteine combined with cysteamine) scavenge the intracellular ROS produced in oocytes and CCs because of the use of oxygen for energy production through mitochondrial oxidative phosphorylation (Deleuze & Goudet, 2010; Rocha-Frigoni *et al.,* 2015). Thus, supplementing media with different types of antioxidants, including enzymatic (superoxide dismutase, glutathione peroxidase, and catalase) and non-enzymatic antioxidant agents (α-tocopherol, ascorbic acid, β-carotene and glutathione, among others), has been proposed to protect oocytes and embryos from oxidative stress during IVP.

Wang *et al*. (2002) reported that vitamins such as tocopherol, ascorbic acid or folic acid play a key role in reducing oxidative damage and improving blastocyst development in mouse embryos. Furthermore, Zullo *et al*. (2016b) demonstrated that the supplementation of culture medium with crocetin, which has a high antioxidant capacity, leads to an improvement of cryotolerance of bovine embryos. In recent years different types of antioxidants have been used during the IVP, for example thiol compounds such as: Cysteamine, which increased GSH levels in bovine oocytes and acts as the main non-enzymatic defense system to reduce ROS levels (Deleuze & Goudet, 2010; Rocha-Frigoni *et al.,* 2016). Carnitine, which is an essential cofactor required for the entry of long-chain fatty acids into the mitochondria for the generation of ATP and prevents the impairment of fatty acid beta-oxidation in mitochondria (Boyacioglu e*t al.,* 2014; Dunning *et al.,* 2014; Wu *et al.,* 2011). Therefore, carnitine acts as an antioxidant that neutralizes ROS, especially superoxide anion, and protects cell organelles, including mitochondria against oxidative damage and induced apoptosis (Mishra *et al.,* 2016; Ye *et al.,* 2010).

Furthermore, Wu *et al*. (2011) have demonstrated that carnitine reduces ROS levels and increases GSH during IVM of porcine oocytes.

Another widely used antioxidant is vitamin C, which protects cells against ROS. Kere *et al*. (2012) reported that vitamin C did not affect nuclear maturation status, whilst Tao *et al*. (2010) showed that L-ascorbic acid promoted polar body extrusion in denuded porcine oocytes. In bovine, Dalvit *et al*. (2005) reported that the addition of vitamin C to the oocyte maturation medium exerted no effect on the maturation rates. Nevertheless, the beneficial effect of vitamin C supplementation may be ascribed to an improved culture condition and/or a better cytoplasmic maturation of the oocytes due to a reduced intracellular oxidative status for embryo development, as demonstrated by Torres *et al*. (2019). In this study, ascorbic acid supplementation in both IVM and IVC media reduces lipid accumulation by increasing lipolysis and suppressing lipogenesis in blastocysts, suggesting an improvement of embryo quality. In the same line, Hu *et al*. (2012) reported that the inhibition of ROS generation by vitamin C during the development of pig embryos was dose dependent and improved the total cell number of produced blastocysts.

In recent years the alternative strategy of using natural plants or their extracts (phenolic compounds, including flavonoids and hydrolysable tannins) as antioxidants in animals has been confirmed to be effective and utilized extensively (Zhong & Zhou, 2013). The antioxidant activities of phenolic compounds are due to their structure and particular ability to donate a hydrogen ion to the peroxy radical generated as a result of lipid peroxidation (Zhong & Zhou, 2013). Quercetin and resveratrol are secondary plant metabolites found in fruits, vegetables, grains and flowers (Kang *et al.,* 2013; Kwak *et al.,* 2012). The use of quercetin and resveratrol to avoid oxidative stress *in vivo* and *in vitro* has been shown in several studies by improving mitochondrial function, promoting CCs expansion and increasing blastocysts development (Wang *et al.,* 2014). Also, it have been demonstrated that quercetin, resveratrol and cysteamine altered the expression of certain developmental related genes (*Bax*, *Blc-2*, *Oct-4*, *Cx-43*, *IGF-1*, *GPx4*, *SOD1* and *Glut-1*) (Mukherjee *et al.,* 2014; Sovernigo *et al.,* 2017; Wang *et al.,* 2014).

1.5. Polymethoxylated flavones

Flavonoids are a family of phytochemicals that exhibit a broad spectrum of pharmacological properties. The highest concentration of olymethoxylated flavones (PMFs) such us tangerine, naringenin and nobiletin are found in the citrus peel and have been shown to impart significant protective biological activities including antioxidant, anticancer, antiviral, anti-

inflammatory, when some PMFs interact with several signaling pathways (ERK, PI3K/AKT, CREB) to promote survival in various cell lines (Manthey & Grohmann, 2001; Moosavi *et al.,* 2015; Whitman *et al.,* 2005). PMFs have multiple beneficial effects. *In vivo* studies showed a direct interaction of PMFs with ROS mainly by activation of *Nrf2* pathway, upregulation of antioxidant enzymes and interaction with metal ions as sources of ROS (Razzaghi-Asl *et al.,* 2013).

Some polyphenols have been studied as an important resource in the neurotrophic ambient by agonistic action on tyrosine kinases receptors (Trk), activation of ERK, and PI3K/AKT pathways and upregulation of antioxidant enzymes. Also, the action of certain flavonoids such as apigenin and ferulic acid, increase cyclic adenosine monophosphate response element-binding protein (CREB) phosphorylation and the antioxidant activity of polyphenols in the *Nrf2* pathway and the upregulation of heme oxygenase-1 that contribute to the neurotrophic activity (Huang *et al.,* 2016; Moosavi *et al.,* 2015). In a recent study, Lim & Song, (2016) demonstrated that naringenin, can support TE migration in porcine embryos, crucial to orchestrate conceptus-uterine interactions during the peri-implantation stage of pregnancy. Nevertheless, further research of these flavonoids on preimplantation embryo development is necessary.

1.5.1. Nobiletin

Nobiletin is a hexamethoxyflavone (chemically known as 5,6,7,8,3´,4´-hexamethoxyflavone) isolated from Citrus fruits, first identified as a class of PMFs, along with other members of the flavonoid's family such as baicalein, luteolin and daidzein. Its bioactivity depends on its chemical structure in which the C atoms of the two methoxy groups in the arene ring are in the same plane, whereas the C atoms of the four methoxy groups linking to the chromene ring are not in parallel (Huang *et al.,* 2016). This conformational characteristic of chiral structure is indicated by the covalent bond rotation between the arene and the chromene rings and the conformational alternations of the methoxy groups (Huang *et al.,* 2016; Noguchi *et al.,* 2016). Due to its structure and lipophilic nature, nobiletin is easily absorbed across the cytoplasmic membranes (facilitated diffusion system) (Figure 8); mechanism similar to that used by other flavonoid substances such as: luteolin, quercetin and tangeretin (Kimura *et al.,* 2014; Singh *et al.,* 2011). However, the details of its mechanisms of action are still unclear. Currently available studies regarding nobiletin have been performed on a pharmacological level in different cell types and animals (Table 1).

Nobiletin has a broad range of biological effects including antioxidation (Choi *et al.*, 2007) and cell cycle regulation (Huang *et al.,* 2016; Lam *et al.,* 2011; Wu *et al.,* 2015). Studies carried out in cell lines culture have demonstrated that nobiletin can modulate signaling cascades, including

ERK1/2 and PI3K/AKT signaling pathways (Huang *et al.,* 2016; Zhang *et al.,* 2016), but the specific mechanism of action through these signaling pathways are not clear yet. Thus, more research on specific intracellular objectives is needed, especially for *in vivo* medical applications.

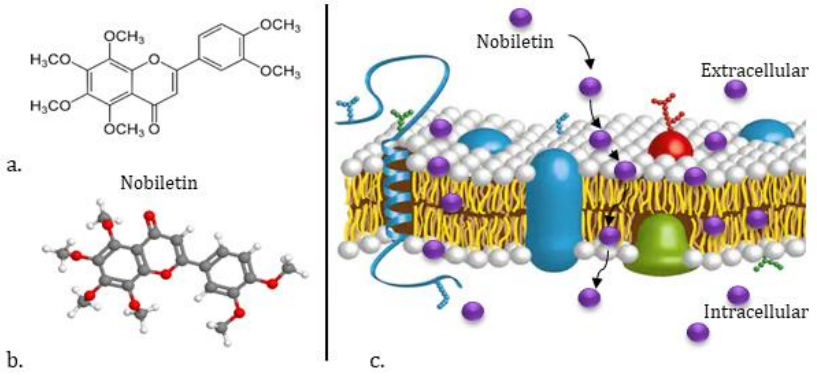

Figure 8. Chemical structure and diffusion mechanism of nobiletin. Chemical structure of Nobiletin (a). 2D and (b). 3D. (c). Model of binding of nobiletin to the membrane and diffusion to the intracellular space through the lipid bilayer due to its lipophilic nature. Adapted and modified from Huang *et al.,* 2016.

Table 1. Biological effects of nobiletin on different cell types and animals

Cell types/animals	Biological functions	References
3T3-L1 preadipocytes	Differentiation↑, p-CREB↓, lipid accumulation↓, GPDH↓, PPARγ↓	Choi *et al.,* 2011
3T3-F442A adipocytes	Glucose uptake↑, PI3K/AKT↑, PKA/CREB signaling↑	Onda *et al.,* 2013
C6 glioma cells	cellular proliferation↓, Ras activity↓, MEK/ERK signaling↓	Aoki *et al.,* 2013
HepG2 cells	Insulin resistance↓, MAPK↑, LDLR↑, DGAT1/2↓, Cpt1α↑, β-oxidation↑	Mulvihill *et al.,* 2011
HFD-induced obese mice	PPARα↑, TNFα↓, GLUT4↑, p-Akt↑	Lee *et al.,* 2013
HT22 cells	p-p38↓, p-JNK↓, Bax↓, caspase-3↓, Bcl-2↑, apoptosis↓	Cho *et al.,* 2015
Human ovarian cancer cells	HIF-1α↓, AKT↓, NF-κB↓, VEGF↓	Chen *et al.*, 2015
Mouse Leydig cells	c-AMP/CREB↑, PDE↓, testosterone production↑	Horigome *et al.,* 2016

PC12 cells	c-FOS↑, CRE-dependent transcription↑, PKA/ERK/MEK/CREB↑	Kimura *et al.,* 2014a
Rats	Hexokinase↑, succinate dehydrogenase↑, mitochondrial metabolism↑	Jojua *et al.,* 2015
Rat I/R injury	cell death↓ CREB↑, Akt↑,	Yasuda *et al.,* 2014
Rat primary astrocytes	iNOS↓, NO production↓, NF-κB↓, p38-MAPK phosphorylation↓	Ihara *et al.,* 2012
U87, Hs683 glioma cells	Cell viability↓, cyclin D1↓, E2 promoter-binding factor 1↓, CDK2↓, CDK4↓, p-PKB↓, p-p38 MAPK↓, p-ERK↓, p-JNK↓	Lien *et al.,* 2016
Zebrafish embryos and HUVECs	G0/G1 arrest↑, apoptosis↓	Lam *et al.,* 2011

1.5.1.1. Nobiletin interaction with several signaling pathways

All research on the interaction of nobiletin with several signaling pathways has been carried out *in vivo* in cell line cultures. One example of these interactions is in glioma cells where nobiletin inhibits the cellular proliferation through suppression of Ras activity and MEK/ERK signaling pathway, that may be related to downregulation of cyclin D1, *CDK*2, phosphorylation levels of p38 MAPK, ERK, and JNK (Aoki *et al.,* 2013; Lien *et al.,* 2016). It has also been shown that nobiletin regulates lipid and glucose metabolism, increases the expression of *PPARα* and *PPARγ* and upregulates the expression of *GLUT4* and AKT phosphorylation (Huang *et al.,* 2016; Lee *et al.,* 2013; Onda *et al.,* 2013). In some cellular lines (PC-3 and DU-145 cells) nobiletin reduces cell viability and attenuates the expression of *VEGF* and *NF-κB* through downregulation of AKT/HIF-1α signaling pathway (Chen *et al.,* 2014) and in rat primary astrocytes cultures inhibits the expression of iNOS and suppresses Nf-kB signaling and p38-MAPK phosphorylation (Ihara *et al.,* 2012). In tumor cells derived from mouse testis, nobiletin enhances testosterone production through the activation of cAMP/CREB signalling by suppressing activation of cyclic nucleotide phosphodiesterase (Horigome *et al.,* 2016). In ovarian cancer cells, nobiletin may significantly inhibit cell viability, proliferation, VEGF protein secretion and promotes cell cycle arrest at G0/G1 phase through downregulation of ERK1/2 and cyclin D1 (Huang *et al.,* 2016). In addition, promotes cell apoptosis through decreasing the expression of *Bcl-xL*, AKT, and mTOR (Chen *et al.,* 2015; He *et al.,* 2015). Lam *et al*. (2011) demonstrated, for the first time, the potent anti-

angiogenic activity of nobiletin through modulation of cell cycle and the VEGF-A pathway in zebrafish embryo. Also, showed that G1 arrest was slightly more pronounced in endothelial cells, possibly indicating a certain selectivity of nobiletin for this cell type, while the apoptosis pattern was not altered (Lam *et al.,* 2011). These results of nobiletin on endothelial cells, are similar to that reported by Morley *et al*. (2007) in breast and colon cancer cell lines, where nobiletin and tangeretin treatments inhibited cancer cells proliferation with accumulation of cells in G1 phase without any effect on apoptosis. In *in vitro* studies, nobiletin has also been shown to suppress angiogenesis in human umbilical vein endothelial cells by inhibiting the phosphorylation of MEK (Miyata *et al.,* 2008).

After all, the biological effect of nobiletin, its association with different cellular signaling pathways, and its capacity of regulating specific genes expression needs to be studied in detail in order to understand the mechanism(s) of action and to identify target receptor molecules.

1.5.1.2. Influence of nobiletin in the PI3K/AKT pathway and in embryo development

So far, the only available report linking the effect of nobiletin with embryonic development has been on zebrafish, demonstrating that nobiletin caused overall growth arrest in these embryos by G1 cell cycle arrest points (Lam *et al.,* 2011). On the other hand, Chen *et al*. (2015), revealed that nobiletin could modulate the progression of ovarian cancer cell lines (OVCAR-3 and CP-70) by PI3K phosphorylation through suppression or mutations in Ras or Raf activity, downregulation of cyclins, important factors during arrests at G0/G1 phase in cell cycle or MEK1 inhibition. In addition, studies with quercetin and naringenin (PMFs similar to nobiletin) showed the capacity of these flavonoids to partially protect embryonic development from oxidative damage in mouse embryos (Pérez-Pastén *et al.,* 2010; Yu *et al.,* 2014), or supports the migration of TE cells by PI3K/AKT signaling pathway (Lim & Song, 2016).

Nevertheless, the mechanism by which nobiletin inhibits or modulates the PI3K/AKT/ERK signaling pathways is not fully understood and to date, there are no studies available on the possibly beneficial effects of nobiletin supplementation on mammalian preimplantation embryo development *in vitro* and on the quality of the produced blastocysts.

JUSTIFICATION AND OBJECTIVES

2. Justification and Objectives

2.1. Justification

IVP is a reproductive biotechnology used widely to increase the number of offspring from superior phenotypes, to treat infertility problems and also to address fundamental questions about endocrine control, molecular switches and metabolic pathways that modulate early embryonic development. However, IVP is a multifactorial process depending on extrinsic and intrinsic factors, which makes necessary research at cellular and molecular levels in order to increase its efficiency (Galli *et al.,* 2003). Today the technique still has limitations, considering that not all oocytes have the ability to develop into a viable embryo after IVM, with only 30% to 40% of mature oocytes reaching the blastocyst stage (Rizos *et al.,* 2002). Although a successful IVM involves a nuclear, cytoplasmic and molecular maturation, necessary for embryonic development (Coticchio *et al.,* 2015*)*, studies have shown that there are certain parameters that may be related with these low embryo production rates such as: oocyte morphology, metabolic pathways that modulate maturation and early embryonic development, quantitative changes of key genes related to quality and development and increase of ROS, the latter caused by the oxygen tension at which IVM is performed (Ambrogi *et al.,* 2017).

Another factor that could also be involved with the low production rates under *in vitro* conditions, is the alteration in the dynamics of embryonic development and the speed of cleavage during compaction, due to morphological and metabolic changes that occur in the first 4 days of development that coincides with the switch from maternal control to EGA (Gad *et al.,* 2012; Graf *et al.,* 2014). During EGA, an increase in intercellular adhesions and embryonic polarization also occurs, leading to the formation of the blastocyst, in which the ICM and TE cells are distinguished (Menchero *et al.,* 2017), and which are important for embryo quality. Furthermore, it has been suggested that apart from the origin of the oocyte and the culture environment affecting EGA (Lonergan & Fair, 2008; Rizos *et al.,* 2008), there is a complex network of signaling pathways responsible for cell division and differentiation, cytoskeletal rearrangements, cell proliferation and even the activation of other signaling pathways necessary for the embryo development, one of them being the pathway of MAPK and PI3K/AKT (Baran *et al.,* 2013; Menchero *et al.,* 2017). Evidence in mice showed that MAPK pathway has a key regulatory role of the cell cycle during meiosis, as well as for the development from 8-16 cell to blastocysts by regulation of filamentous actin (Natale *et al.,* 2004). However, the same group demonstrated that bovine embryos require both p38 MAPK and ERK signalling for their development to the blastocyst stage (Madan *et al.,* 2005). Instead the PI3K/AKT signaling pathway is fundamental in cell proliferation, survival, migration and metabolism and its inhibition can cause a significant delay in blastocyst hatching

(Manning & Cantley, 2007; Riley *et al.,* 2005; Zhang *et al.,* 2007). Cecconi *et al*. (2010) demonstrated in a mouse study that AKT is expressed from 1-cell stage to morula on cell surface, while at blastocyst stage is present in the TE cells. Recently, it was confirmed that AKT may be indispensable for the first cell lineage differentiation of mouse (Xu *et al.,* 2019) and in cattle it has been shown that inhibition of AKT signaling reduces the developmental competence of early bovine embryos suggesting a potential requirement of AKT for bovine early embryonic development (Ashry *et al.,* 2018).

In this context, to improve developmental rates *in vitro* and the quality of produced blastocysts, several studies have proved the addition of different types of natural antioxidants such as: resveratrol, crocetin or ascorbic acid (Mishra *et al.,* 2016; Rocha-Frigoni *et al.,* 2016). The use of antioxidants of plant origin (phenolic compounds, including flavonoids) has been implemented successfully in male and female animal reproduction (Zhong & Zhou, 2013) as well as in IVP by improving mitochondrial function in oocytes and embryos (Ambrogi *et al.,* 2017; Yu *et al.,* 2014) promoting the expansion of CCs (Tao *et al.,* 2010), enhancing embryo development and quality of the produced blastocysts through the interaction with genes related to metabolism, cell division and apoptosis (*Bax, Blc-2, Oct-4, IGF-1, GPx4, SOD1*)(Torres *et al.,* 2018; 2019). Some flavones, such as naringenin and nobiletin, have been shown to play significant protective biological functions, including antioxidant properties, and it has been demonstrated that they interact with several signaling pathways (ERK, PI3K/AKT, CREB) to promote survival in various cell lines (Huang *et al.,* 2016; Moosavi *et al.,* 2015; Whitman *et al.,* 2005), protect embryonic development from oxidative damage (Pérez-Pastén *et al.,* 2010; Yu *et al.,* 2014) or support migration of TE cells by PI3K/AKT signaling pathway (Lim & Song, 2016). Although there have been positive responses with the addition of antioxidant compounds in preimplantation embryonic development, it is not yet clear which antioxidant is the most effective in supporting the development and quality of bovine embryos without altering the activation of the signaling pathways or the EGA.

Having said that, we are still far from a proper use of antioxidant substances supplementation to culture media *in vitro* and their effect on the activation of signaling pathways involved in early embryo development, which would improve our current IVP systems. Thus, the current challenge is the discovery of the most suitable antioxidant that could enhance the development of high-quality embryos *in vitro*.

2.2. Objectives

Nobiletin, a class of polymethoxylated flavone identified from the citrus peel (chemically known as 5,6,7,8,30,40 hexamethoxyflavone) interacts with several signalling pathways (ERK,

PI3K/AKT, CREB) to promote survival in various cell lines and has a broad range of biological effects, including cell cycle regulation, reduction of apoptosis and antioxidation, which are important for the success of oocyte maturation and early embryo development. Therefore, the main objective of this thesis is to **study the effect of nobiletin during the maturation of oocytes and in the IVC during the two main phases of EGA for embryonic development and determine if its mechanism of action is related to the PI3K/AKT signalling pathway**. To achieve this general objective, several specific objectives have been addressed to in three experiments:

Experiment 1:

In vitro maturation increases the production of ROS caused by the oxygen tension at which IVM is performed. Studies have demonstrated that the addition of natural antioxidants in IVM have increased embryonic development and improved blastocysts quality by reducing ROS levels. In recent years, there have been promising results with compounds of natural origin, however, it is not yet clear which antioxidant could be the most efficient to support the development, production, and quality of bovine embryos. Therefore, the objective of this study is **to evaluate the antioxidant activity of nobiletin during IVM on matured bovine oocyte quality and its developmental competence**. The parameters evaluated were:

- Nuclear maturation (meiotic progression to M-II)
- Cytoplasmic maturation:
    - Cortical granules migration
    - Mitochondrial activity and distribution pattern
- Intracellular ROS and GSH levels
- Steroidogenesis of cumulus cells
- Oocyte developmental competence to blastocyst stage
- Quantitative changes of gene expression in matured oocytes, their CCs and produced blastocysts

Experiment 2:

IVC of bovine blastocysts is one of the most important processes in the IVP, since post-fertilization culture conditions can dramatically alter the quality of the embryos and can lead to a failed embryonic development, increasing ROS levels and altering the dynamics of embryonic development, since the first 4 days of preimplantation development are the most important ones

and they coincide with the change from maternal control to the activation of the EGA. In the bovine embryo, this occurs in two different forms: (i) minor EGA (MNEGA) (2 to 8 cell stage) where zygotes and early embryos are transcriptionally and translationally active; (ii) major EGA (MJEGA) (8 to 16 cell stage) that includes a gradual degradation of mRNA molecules of maternal origin and activation of transcription of the embryonic genome (Gad *et al.,* 2012; Graf *et al.,* 2014). However, for the embryos to be produced with good quality, different researchers have suggested that, in addition to the origin of the oocyte, the culture environment and the use of antioxidants, there is a complex network of signaling pathways responsible for cell division and differentiation and even some signaling pathways that may be activated by growth factors produced by the developing embryo itself during EGA. Therefore, the aim of this study is **to evaluate whether supplementation of nobiletin to the *in vitro* culture medium during the two EGA phases improves embryonic development and blastocyst quality and if its action is related to the PI3K/AKT signalling pathway**, in terms of:

- Embryo development:
    - Cleavage rate
    - Blastocysts yield Day 7/Day 8
- Embryo quality
    - Lipid accumulation
    - Mitochondrial activity
    - Total cell numbers
- Quantitative changes of key genes related to quality and development in 8-cell, 16-cell embryos and blastocysts.
- Immunolocalization of phospho-AKT (p-AKT) level
- Western blot analysis for AKT and p-AKT (Thr308/Ser 473).

Experiment 3:

During preimplantational embryo development, a variety of signaling pathways regulate and mediate cellular responses including cell proliferation, growth, and differentiation such as PI3K/AKT signaling pathway that regulates cell survival and proliferation in various cellular types. In recent years, several studies demonstrated that nobiletin has a broad range of biological effects including antioxidation (Choi *et al.,* 2007) and is associated with modulation of some cellular signaling pathways (PI3K/AKT, ERK, NF-kB) that can regulate cell proliferation, cell survival and cell cycle progression through G0/G1 (Huang *et al.,* 2016; Wu *et al.,* 2015), but its molecular mechanism of action is still unclear. Therefore, the aim of this study is to **establish**

whether, when the AKT cascade is inhibited using inhibitors III and IV, nobiletin supplementation to the *in vitro* culture media during the minor (2 to 8-cell stage) or major (8 to 16-cell stage) phase of EGA is able to modulate the development and quality of bovine embryos. The parameters evaluated were:

- Kinetics of embryo development
- Quantitative changes of key genes related to quality and development
- Immunolocalization of phospho-AKT (Thr308)
- Analysis of phospho-rpS6 (phospho Ser235/236) level by western blot.

MATERIALS AND METHODS

3. Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA)

3.1. Oocyte collection and *in vitro* maturation

Immature COCs were obtained by aspirating follicles (2 – 8 mm diameter) from the ovaries of mature heifers (i.e. at least one corpus luteum or remained scars from previous ovulations in one or both ovaries) collected at local slaughterhouses. Only oocytes with a compact, non-atretic cumulus of at least three layers and homogeneous cytoplasm (Class 1 and class 2 COCs) were selected and matured in groups of 50 COCs per well for 24 h in 500 µL of maturation medium, (TCM-199) supplemented with 10% (v/v) FCS and 10 ng/mL epidermal growth factor at 38.5 °C under an atmosphere of 5% CO_2 in air, with maximum humidity.

3.1.1. Assessment of oocyte cytoplasmic maturation

3.1.1.1. Cortical granules distribution

Visualization of CGs distribution was performed according to Arias-Álvarez *et al*. (2009), with minor modifications. Briefly, *in vitro* matured COCs from each treatment were first suspended in 100 µL of PBS without calcium or magnesium supplemented with 0.1% polyvinylpyrrolidone (PVP) and their CCs were removed by gentle pipetting. Next, oocytes were treated with 0.5% (w/v) pronase to digest the zona pellucida. Zona-free oocytes were washed in PBS + 0.1% PVP three times and fixed in 4% (w/v) buffered neutral paraformaldehyde (PF) solution (pH 7.2 – 7.4) for 30 minutes (min) at room temperature and then treated with permeabilization solution (0.02% v/v Triton X-100 in PBS + 1% BSA for 10 min). The oocytes were then treated for 30 min with blocking solution (7.5 % w/v BSA in PBS) and incubated in 100 µg/mL FITC-labeled Lens culinaris (LCA-FITC, Vector Laboratories, Burlingame, USA) for 30 min at room temperature in a dark chamber. Following, oocytes were treated for 30 min with Hoechst 33342 (10 µg/mL) to evaluate nuclear maturation. After staining, oocytes were washed in PBS + 0.1% PVP, mounted in 3.8 µL of mounting medium between a coverslip and a glass slide and sealed with nail polish. Slides were examined using a laser-scanning confocal microscope (Leica TCS SP2) equipped with an argon laser excited at 488 nm and whose detection spectrum is 515 nm.

CGs distribution was analyzed and classified as: non-migrated (CGs distributed throughout the cytoplasm); partially migrated (CGs dispersed and partly clustered throughout the cortical area); and migrated (small CGs arranged at the periphery or adjacent to the plasma membrane)

(Ferreira *et al.,* 2009; Hosoe & Shioya, 1997). Simultaneously, oocytes were evaluated for nuclear maturation.

3.1.1.2. Mitochondrial distribution patterns and quantification of mitochondrial activity

Briefly, *in vitro* matured COCs from each treatment were first suspended in 100 µL PBS + 0.1% PVP and their CCs were removed by gentle pipetting. Next, oocytes were equilibrated for 15 min in maturation medium and then placed in four-well culture plates containing 500 µL of 400 nM MitoTracker DeepRed (Molecular Probes, Eugene, USA) per well. The plates were incubated at 38.5 °C, 5% CO_2 in the dark, and humidified atmosphere for 30 min. The stained oocytes were washed twice in PBS + 0.1% PVP and fixed in 4% PF for 30 min at room temperature. Following, oocytes were treated for 30 min with Hoechst 33342 (10 µg/mL) for evaluating nuclear maturation. After that, oocytes were washed in PBS + 0.1% PVP, mounted in 3.8 µL of mounting medium between a coverslip and a glass slide and sealed with nail polish. Slides were examined using a laser-scanning confocal microscope (Leica TCS SP2) equipped with an argon laser excited at 644 nm with a detection spectrum of 625 – 665 nm. The format, laser, gain, and offset were kept constant for every sample. Serial sections of 5 µm were made for each oocyte and a maximum projection was accomplished for each.

The distribution was classified as: non-migrated (when mitochondria were homogeneously distributed throughout the cytoplasm); partially migrated (mitochondria were heterogeneously distributed throughout the cytoplasm) and migrated (mitochondria were distributed with granular aggregations) (Ferreira *et al.,* 2009; Lima *et al.,* 2018; Zhang *et al.,* 2018; Zhao *et al.,* 2011). For the assessment of mitochondrial activity, the fluorescence signal intensity (pixels) was quantified. Images obtained were evaluated using the ImageJ program (NIH; http://rsb.info.nih.gov/ ij/), using the freehand selection tool. Fluorescence intensity in each oocyte was determined using the following formula: Relative fluorescence = integrated density (IntDen) - (area of selected oocyte x mean fluorescence of background readings). Fluorescence intensities are expressed in arbitrary units (a.u.) (Hamdi *et al.,* 2018; Rocha-Frigoni *et al.,* 2016). Simultaneously, these oocytes were evaluated for nuclear maturation.

3.1.2. Assessment of oocyte nuclear maturation

Matured oocytes from all treatments stained for CGs distribution and mitochondrial distribution and activity were also stained with Hoechst 33342 solution (10 µg/mL of PBS) for nuclear chromosomal and polar body evaluation. Oocytes were classified as follows: immature oocytes comprising the stages of germinal vesicle (GV, nucleus well defined), germinal vesicle

breakdown (GVBD, chromosome condensation), metaphase-I (M-I, first metaphasic plate visible); and matured oocytes comprising the stage of metaphase-II (M-II, represented by the presence of the first polar body and/or the second metaphasic plate). Nuclear maturation was assessed under an epifluorescence microscope (Nikon 141731) equipped with a fluorescent lamp (Nikon HB-10104AF) and UV-1 filter. Oocytes in M-II were considered as matured.

3.1.3. Levels of reactive oxygen species and glutathione

For evaluation of ROS and GSH, *in vitro* matured COCs, were first suspended in 100 µL PBS + 0.1% PVP and their CCs were removed by gentle pipetting, then were incubated in four-well plates containing 500 µL of 10 µM of CellROX Deep Red Reagent (Invitrogen, Eugene, USA) for ROS and 20 µM of CellTracker Fluorescent (Invitrogen) for GSH per well, at 38.5 °C, 5% CO_2 in a dark and humidified atmosphere for 30 min. After staining, oocytes were washed twice with PBS+ 0.1% PVP, mounted in 3.8 µL of mounting medium between a coverslip and a glass slide, sealed with nail polish, and were imaged immediately using an epifluorescence microscope (Nikon 141731). Fluorescence emitted from the oocytes was captured using B-2E/C (ROS) and UV-2A (GSH) filters for ten seconds after exposure to UV light. The digital images were processed and analyzed using ImageJ. The relative ROS and GSH fluorescence intensity in each oocyte were assessed as described for the mitochondrial activity (Section 3.1.1.2).

3.1.4. Steroidogenic production of estradiol and progesterone by cumulus cells

Progesterone (P_4) concentration was measured in spent maturation media by solid-phase radioimmunoassay method (RIA) using the methods as described by Santiago-Moreno *et al.* (2005). Aliquots of 100 µL were used in duplicate, then each of the samples was measured in the liquid Scintillation Counter (Tri-Carb® 2100TR) including the measurement of the standard curve. The intra-assay coefficient of variation was 11% and assay sensitivity was 0.4 ng/mL. Estradiol (E_2) concentrations in spent maturation media were measured by a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding specific kit (DEH3355 DEMEDITEC Diagnostics GmbH, Kiel, Germany) according to the manufacturer´s instructions. Intra-assay coefficients of variation were 6%. Results are expressed as average E_2 (pg/mL) and P_4 (ng/mL) concentrations produced by 50 COCs after the IVM period using 3 replicates.

3.2. Sperm preparation and *in vitro* fertilization

IVF was performed as described previously (Lopera-Vasquez *et al.,* 2017). Briefly frozen semen straws (0.25 mL) from an Asturian Valley bull previously tested for IVF were thawed at 37 ºC in a water bath for 1 min and centrifuged for 10 min at 280 × *g* through a gradient of 1 mL of

40% and 1 mL of 80% Bovipure (Nidacon Laboratories AB, Göthenborg, Sweden) according to the manufacturer´s instructions. The sperm pellet was isolated and washed in 3 mL of Boviwash (Nidacon Laboratories AB) by centrifugation at 280 × *g* for 5 min. The pellet was re-suspended in the remaining 300 µL of Boviwash. The final concentration of spermatozoa was adjusted to $1x10^6$ spermatozoa/mL. Gametes were co-incubated for 18 - 22 h in 500 µL fertilization media (Tyrode's medium) with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate and 6 mg/mL fatty acid-free BSA supplemented with 10 mg/mL heparin sodium salt (Calbiochem) in four-well cell culture plates in groups of 50 COCs per well under an atmosphere of 5% CO_2 in the air, with maximum humidity at 38.5 °C.

3.3. *In vitro* culture of presumptive zygotes

At 18 - 22 h post-insemination (hpi), presumptive zygotes were denuded of CCs by vortexing for 3 min and then cultured in groups of 25 in 25 µL droplets of culture medium (SOF; (Holm *et al.,* 1999)) with 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 µL/mL BME amino acids, 10 µL/mL minimum essential medium (MEM) amino acids and 1 µg/mL phenol red supplemented with 5% FCS under mineral oil (only Experiment 1) at 38.5 °C under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 with maximum humidity. Due to the lipophilic nature of nobiletin in Experiments 2 and 3, the embryos were cultured in a four-well dish containing 500 µL per well of culture medium, depending on the experiment, zygotes were cultured in SOF + 5% FCS, supplemented or not with nobiletin (Experiment 2) or supplemented with nobiletin, or AKT inhibitors, or nobiletin + AKT inhibitors (Experiment 3).

3.3.1. Assessment of embryo development and quality

3.3.1.1. Embryo development

Depending on the experiment:

- Experiment 1: The cleavage rate was recorded at day 2 (48 hpi) and cumulative blastocyst yield were determined on Days 7 and 8 pi under a stereomicroscope.
- Experiment 2 and 3: Developmental rate was recorded at 54 hpi from MNEGA and MJEGA (≥8-cell) and at 96 hpi from MJEGA phase (≥16-cell). For both phases, cumulative blastocyst yields were recorded at Day 7, and 8 pi under a stereomicroscope.

3.3.1.2. Embryo quality

Only in Experiment 2, in blastocysts we assessed the lipid content and mitochondrial activity.

3.3.1.2.1. Lipid content quantification.

Day 7 blastocysts (~30 per group) were simultaneously evaluated regarding quantity of lipid droplets and total cell number. Blastocysts were first suspended in 100 µL PBS without calcium or magnesium supplemented with 0.1% PVP. Next, blastocysts were fixed in 4% PF for 30 min at room temperature and permeabilized with 0.1% saponin for 30 min and stained for 1 h with 20 µg/mL Bodipy 493/503. Cell nuclei were counterstained with Hoechst 33342 (10 µg/mL) for 30 min, washes in PBS + 0.1% PVP three time for 5 minutes each and then mounted in 3.8 µL of mounting medium between a coverslip and a glass slide which was sealed with nail polish. Slides were examined using a laser-scanning confocal microscope (Leica TCS SP2) equipped with an argon laser excited at 488 nm and with an emission spectrum of 500 - 537 nm for visualization of lipid droplets. All images were captured using the same parameters, performing sequential acquisition.

The lipid quantification in blastocysts was obtained by analysis of the total area of lipids in each blastocyst and obtaining the average of three sections. We captured three images of each blastocyst: one in the middle of the blastocyst (the image with largest diameter) and the other two in the middle of the resulting halves. We used a 63× objective at a resolution of 1024 × 1024 and images were analyzed used the 'nucleus counter' tool, set to detect, distinguish and quantify droplet areas with the ImageJ program. For blastocysts, lipid quantity was corrected by area, to account for varying blastocyst sizes. After verification of a significant correlation ($r^2 = 0.84$ and $P < 0.0001$ by Pearson's correlation test) between lipid quantity of three sections in 30 blastocysts (10 per group) we chose the section with the largest area per embryo to be analyzed *(Collado et al.,* 2015). Simultaneously, the number of cells per blastocyst was determined by counting the Hoechst-stained cells under an epifluorescence microscope (Nikon 141731) equipped with a fluorescent lamp (Nikon HB-10104AF) and UV-1 filter.

3.3.1.2.2. Mitochondrial activity measurement

The mitochondrial activity in the blastocysts were assessed as described for the mitochondrial activity (Section 3.1.1.2).

3.3.1.2.3. Differential staining of blastocysts

Only in Experiment 3c, differential staining of ICM and TE cells was carried following the procedures of Bermejo-Alvarez *et al*. (2012) with minor modifications. Blastocysts from all experimental groups (Control; C_{DMSO}; Inh III; Inh IV; Nob; Nob+Inh III; and Nob+Inh IV) had their zona removed with 0.5% (w/v) pronase in PBS. Zona-free embryos were washed in PBS three times and were then fixed in 4% PF in PBS supplemented with 1% BSA (PBS+1% BSA) for 10 min

at room temperature. After fixation, embryos were washed three times in PBS+1% BSA and kept in that medium at 4 ºC until analysis. For immunostaining, cells were permeabilized in PBS + 5% goat serum (IF buffer) with 1% Triton X-100 for 45 min at room temperature. Blastocysts were then incubated overnight at 4 ºC in primary antibody solution consisting of PBS+1% BSA, 20% IF buffer and 1:1000 mouse monoclonal anti-CDX2 antibody (Biogenix, Fremont, CA, USA). Following incubation, blastocysts were washed twice in PBS+1% BSA and incubated in the secondary antibody solution consisting of PBS+1% BSA, 20% IF buffer, 1:3000 Alexa Fluor goat anti mouse 488 (Invitrogen, Grand Island, NY, USA) and Hoechst 33342 (10 μg/mL) for 2 h at room temperature. Finally, embryos were washed three times in PBS+1% BSA and mounted in 3.8 μL of mounting medium between a coverslip and a glass slide and sealed with nail polish. Stained blastocysts were analyzed by widefield fluorescence microscope ith structured illumination (ApoTome, Zeiss Microscopy GmbH, Jena, Germany). Z-stack sections of 5 μm were taken in the 405 nm (Hoechst positive cells, total cell number) and 488 nm (CDX2-positive cells, TE cells) channels. Images obtained were evaluated using the ImageJ program (NIH, ImageJ version 1.52k software (http://rsbweb.nih.gov/ij/)).

3.3.2. Gene expression analysis

For the gene expression analysis, the following groups of samples are prepared according to the experiment:

- Experiment 1: three pools of 10 oocytes and their corresponding CCs and Day 7 blastocysts per treatment group.
- Experiment 2a: three independent pools of 10 embryos per stage (8-cell, 16-cell, and Day 7 blastocyst) obtained from each experimental group cultured with or without nobiletin during MNEGA or MJEGA).
- Experiment 3b: Embryos of 8-cell (MNEGA) and embryos of 16-cell (MJEGA), were collected in three pools of 10 per treatment group,

Poly(A) RNA was extracted using the Dynabeads mRNA Direct Extraction Kit (Ambion; Thermo Fisher Scientific) with minor modifications (Bermejo-Álvarez *et al.,* 2008). Immediately after poly(A) RNA extraction, reverse transcription (RT) was performed using an Moloney murine leukemia virus (MMLV) Reverse Transcriptase 1st-Strand cDNA Synthesis Kit according to the manufacturer's instructions (Epicentre Technologies Corp, Madison, WI, USA). Tubes were heated to 70 ºC for 5 min to denature the secondary RNA structure, allowing Poly(T) random primers and Oligo dT annealing, and the RT mix was then completed by adding 0.375 mM dNTPs

(Biotools, Madrid, Spain), 6.25 U RNAsin RNAse inhibitor (Promega, Madison, WI, USA), MMLV HP RT 10x reaction buffer, 5 mM DTT and 5 U MMLV high-performance reverse transcriptase (Epicentre Technologies Corp, Madison, WI, USA). Samples were incubated at 25 ºC for 10 min, and then at 37 ºC for 60 min, to allow the RT of RNA, and finally at 85 ºC for 5 min to denature the enzyme. All mRNA transcripts were quantified in duplicate using a Rotorgene 6000 Real-Time Cycler (Corbett Research, Sydney, Australia). RT–quantitative polymerase chain reaction (qPCR) was performed by adding a 2 µL aliquot of each cDNA sample (∼60 ng µL-1) to the PCR mix (GoTaq qPCR Master Mix, Promega) containing specific primers to amplify the genes of interest. Primer sequences are provided in Table 2. The selection of genes to be evaluated was carried out considering expression of key genes in preimplantation embryonic development. All primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries when possible. For quantification, RT-qPCR was performed as described previously (Bermejo-Alvarez *et al.,* 2010). The PCR conditions were tested to achieve efficiencies close to 1. Relative expression levels were quantified by the comparative cycle threshold (CT) method (Schmittgen & Livak, 2008). Values were normalized using two housekeeping genes: *H2AFZ* and *ACTB*. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the mean CT value of the two housekeeping genes from the CT value of the gene of interest in the same sample. The calculation of ΔΔCT involved using the highest treatment ΔCT value (i.e. the treatment with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold-changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

Table 2. Primers used for RT-qPCR

Gene symbol	Primer sequence (5'- 3')	Fragment size (bp)	GenBank accession number
ABCB1	GAAACGAAGTTAAGATCTTGAAGGGCC CCGTCGATACTGACCATGCCC	153	XM_024991021
ACTB	GAGAAGCTCTGCTACGTCG CCAGACAGCACCGTGTTGG	264	AF191490.1
BCL2	TGGAGCAGGTGCCTCAGGA ATCTCGAAGGAAGTCCAGCGTC	300	NM_001166486.1
BMP15	ATCATGCCATCATCCAGAACC TAAGGGACACAGGAAGGCTGA	72	NM_001031752.1

BMP7	AACCATGCCATCGTGCAGACGC AAGCCCGGACAACCATGTTTGC	250	NM_001206015.1
CDH1	GATTGCAAGTTCCCGCCATC ACATTGTCCCGGGTGTCATC	144	NM_001002763
CDK2	TCTTTGCTGAGATGGTGACCC GTTAGGGTCGTAGTGCAGCAT	242	NM_001014934.1
CDX2	ATCACCATCCGGAGGAAAGC CTCATGGCTCAGCCTGGAAT	333	NM_001206299.1
CHD1	AACGGCAGGAATCCCAGAAT AATAACTGTCCCTGCTGCTGT	123	XM_019965464.1
CLIC1	CCATTCCGGATGTGTTTCGTGG GAAACCACCCAGGGCCTTTGTG	196	NM_001015608.1
CYP51A1	GGCCCAAGGTGATTTCCATTTC CTCCCAAGAAACCCTGCACTGG	168	BC149346.1
DNMT3B	AGGGAGTCGACAGCCTAATCA CTCTTGGACAGTCGTGAGCTT	202	NM_181813.2
FOSL1	CTCTCCTACTACCACTCACC GTACTAGCCATTGTAGGTCC	153	NM_182786.2
GAPDH	ACCCAGAAGACTGTGGATGG ATGCCTGCTTCACCACCTTC	247	BC102589
GDF9	AGCGCCCTCACTGCTTCTATAT TTCCTTTTAGGGTGGAGGGAA	80	NM_174681.2
GJA1	TGCCTTTCGTTGTAACACTCA AGAACACATGAGCCAGGTACA	142	NM_174068.2
GPX1	GCAACCAGTTTGGGCATCA CTCGCACTTTTCGAAGAGCATA	116	NM_174076.3
H2AFZ	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	212	NM_016750
H3-3A	TTTTTCCATGGGGTCAAAAG TGGAAAAACTGCCAATACCTG	96	NM_001014389.2
H3-3B	GTGGTGGGGAGTGTTGTCTT AACGCGCAAAGCATTTTACT	99	NM_001242571.2
IGF2R	GCTGCGGTGTGCCAAGTGAAAAAG AGCCCCTCTGCCGTTGTTACCT	201	NM_174352.2
KLF4	AGGTGAGAAACCTTACCACTG CCATGATTGTAGTGCTTTCCA	291	NM_001105385.1
MAPK1	GTCGCCATCAAGAAAATCAGC GGAAGGTTTGAGGTCACGGT	308	NM_175793

NFE2L2	CAGGACATTGAGCAAGTTTGG GTGGAAAGGATGCTGTTGAAG	234	NM_001011678.2
POU5F1	CGAGTATCGAGAACCGAGTG CAGGGTTCTCTCCCTAGCTC	440	NM_174580.1
PPARα	CAACCCGCCTTTCGTCATCCAC TCCGCCTCCTTGTTCTGGATGC	102	NM_001034036.1
PPARGC1A	AAAAGCCACAAAGACGTCCG TCTGCTGCTGTTCCGGTTCT	111	NM_177945
RPS6KB1	GGGATAGAGCAGATGGACGTG TGGTCGTTTGGAGATCATGGG	117	NM_205816.1
SOD2	GCTTACAGATTGCTGCTTGT AAGGTAATAAGCATGCTCCC	101	S67818.1
TEAD4	AGTTCTCTGCCTTCTTGGAGC TTCTTCTCGGGGAACTTGTCG	151	XM_010805630.3

3.3.3. Immunofluorescence of phosphorylated-AKT (p-AKT)

Immunolocalization of p-AKT was performed depending the experiment in:

- Experiment 2a: Day 7 blastocysts (n=10 per group)
- Experiment 3a: 8-cell (n=15 per group), 16-cell embryos (n=15 per group), and Day 7 blastocysts (n=10 per group)

Immunolocalization of p-AKT was performed according to López-Cardona *et al*. (2016) with minor modifications. Samples were washed twice with PBS + 0.1% PVP and fixed in 4% PF for 10 min at room temperature. Next, they were permeabilized by incubation in PBS with 10% FCS and 1% Triton X-100 for 45 min at room temperature. After permeabilization, samples were incubated overnight at 4 ºC in PBS + 0.1% PVP and 5% FCS and 1:100 rabbit polyclonal antibody against p-AKT (Thr308/Ser473) (D9E) XP® Rabbit mAb (Cell Signaling Technology, #4060). Following incubation, samples were washed twice in PBS + 0.1% PVP and incubated in PBS supplemented with 5% FCS and 1:250 goat anti-rabbit polyclonal antibody Alexa Fluor 488-conjugate (Molecular Probes, Eugene, OR, USA), for 2 h at room temperature followed by washing again three times in PBS + 0.1% PVP. In all cases, nuclei were stained with Hoechst 33342 (10 µg/mL). Finally, samples were mounted in microdrops with Fluoromount G (EMS, Hatfield, UK) and examined by an apotome microscopy (Leica TCS-SPE). Negative control was prepared to omit the primary antibody before adding the secondary antibody.

3.3.4. Western blot analysis

To investigate the effect of nobiletin during IVC on AKT phosphorylation, in bovine embryos the following groups of samples are prepared according to the experiment:

- Experiment 2c: Day 7 blastocysts (n=20 blastocysts/group, n = 3 replicates/EGA phase) were collected and subjected to western blott analysis for: (i) total (t)AKT; (ii) p-AKT (residues: Thr308 and Ser473) and (iii) actin as loading control.
- Experiment 3d: Embryos of 8-cells and 16-cells (MNEGA and MJEGA, respectively) (n=30 embryos/treatment, n=3 replicates/EGA phase) were collected and subjected to western blot analysis for p-rpS6 and (iii) actin as loading control.

The western blot analysis was performed as described previously by Ashry *et al*. (2015) with minor modifications. Samples were lysed in 1× RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris [pH 7.6]), supplemented with 1× protease, phosphatase Inhibitor Cocktail (Roche, Basel, Switzerland), for 1 h at 4 ºC. The samples were mixed with 1× sample buffer and then denatured at 95 ºC for 10 min. Proteins were resolved by SDS-PAGE (12% acrylamide gel loading 45 µL of total protein per well) and transferred onto a nitrocellulose membrane for immunoblotting following standard procedures. After the transfer, membranes were blocked for 30 min in 3% BSA in PBS + 0.1% Tween-20 (PBS-T) at room temperature, and was incubated overnight at 4 ºC with AKT rabbit polyclonal antibody [1:1000 (Vol:Vol), Cell Signaling Technology, #9272S]; or p-AKT (Thr308) polyclonal antibody [1:1000 (Vol:Vol), Cell Signaling Technology, #9275S]; or p-AKT (Ser473) polyclonal antibody [1:1000 (Vol:Vol), Cell Signaling Technology, #9271S] (Experiment 2c) and p-rpS6 (phospho Ser235/236) rabbit polyclonal antibody [1:1000 (Vol:Vol), GeneTex Inc., Irvine, USA, GTX130430] (Experiment 3d). Then, incubation with the secondary antibody goat anti-rabbit IgG-HRP [1:2500 (Vol:Vol), Cell Signaling Technology, #7074S] was conducted for 2 h at room temperature revealed by Enhanced Chemiluminescence kit (RPN2109, ECLTM, Amersham GE Healthcare) and detected by an ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare Life Sciences, USA, 29005063). The monoclonal anti-β-actin–peroxidase antibody produced in mouse was used as the loading control.

For this purpose, after the chemiluminescent detection of the phosphorylated antibody, membranes were stripped by washing extensively in TBS-T, three times for 10 minutes each, and repeating the blocking step, and then the membranes are re-probed with anti-actin antibody or the other antibodies. In all cases, intensities of protein bands ((optical density (OD)) were

quantified by ImageJ software and normalized relative to the abundance of actin in each lane; the ratio of the OD of the protein concerned (AKT/p-AKT or rpS6) in relation to actin is presented in the form of bar charts.

3.4. Statistical Analysis

All statistical tests were performed using the software package SigmaStat (Systat Software Inc., San Jose, CA, USA). Nuclear maturation, CG and mitochondrial distribution patterns, mitochondrial activity, ROS and GSH measurements, steroidogenic production, cleavage rate, blastocysts yield, lipid content, number of cells, embryo cell number (ICM, TE) and relative mRNA abundance were normally distributed with homogeneous variance, so one-way analysis of variance (ANOVA), with arcsine data transformation, followed by Tukey´s test, was performed to evaluate the significance of differences between groups. For lipid quantification in blastocysts, the correlation was determined by Pearson's correlation coefficient test. Values were considered significantly different at $P<0.05$. Unless otherwise indicated, data are presented as the mean ± s.e.m.

3.5. Experimental design

3.5.1. Experiment 1: Effect of nobiletin supplementation in IVM of bovine oocytes and their developmental competence and quality of produced blastocysts

In this experiment the developmental capacity of bovine zygotes and the quality of the produced embryos were assessed on Day 7-8 after *in vitro* maturation of the oocytes under the following conditions: TCM-199 + 10% FCS + 10 ng/mL EGF (Control group) (used as a basic medium for the remaining groups) supplemented either with 10, 25, 50 and 100 μM nobiletin (MedChemExpress, MCE, Sweden); (Nob10, Nob25, Nob50 and Nob100, respectively) or dimethyl sulfoxide (DMSO control (CDMSO), 0.01% DMSO vehicle for nobiletin dilution) (For details see Figure 9). The concentration of nobiletin was based on the findings of other studies in which this polymethoxylated flavonoid was used *in vivo* in zebrafish and chick embryos and *in vitro* in human umbilical vein, showing an anti-angiogenic activity at concentrations between 30 and 100μM (Kunimasa *et al.,* 2010; Lam *et al.,* 2011; Qu *et al.,* 2018).

After 24 h of IVM, matured COCs under different conditions were employed to evaluate: nuclear maturation (Control: n=117; CDMSO: n=122; Nob10: n=133; Nob25: n=146; Nob50: n=149; Nob100: n=144), mitochondria distribution patterns and mitochondrial activity (Control: n=59; CDMSO: n=56; Nob10: n=61; Nob25: n=76; Nob50: n=71; Nob100: n=74), CGs migration (Control: n=58; CDMSO: n=66; Nob10: n=72; Nob25: n=70; Nob50: n=78; Nob100: n=70), levels of ROS and GSH (Control: n=54; CDMSO: n=48; Nob10: n=50; Nob25: n=47; Nob50: n=53; Nob100: n=49). For all consecutive experiments, overall cleavage rate was recorded at 48 hpi and blastocyst development was recorded on Day 7 and 8 pi. For mRNA abundance of selected genes (oocytes, their CCs and blastocysts Day 7), pools of 10 per treatment group were collected (three replicates), were washed in fresh PBS and snap frozen in LN_2 and stored at -80 ºC until mRNA extraction. In addition, the spent maturation media of three replicates were collected and P_4 and E_2 concentration was measured by solid-phase radioimmunoassay method (RIA) and ELISA (P_4 and E_2, respectively).

A total of eight replicates were carried out.

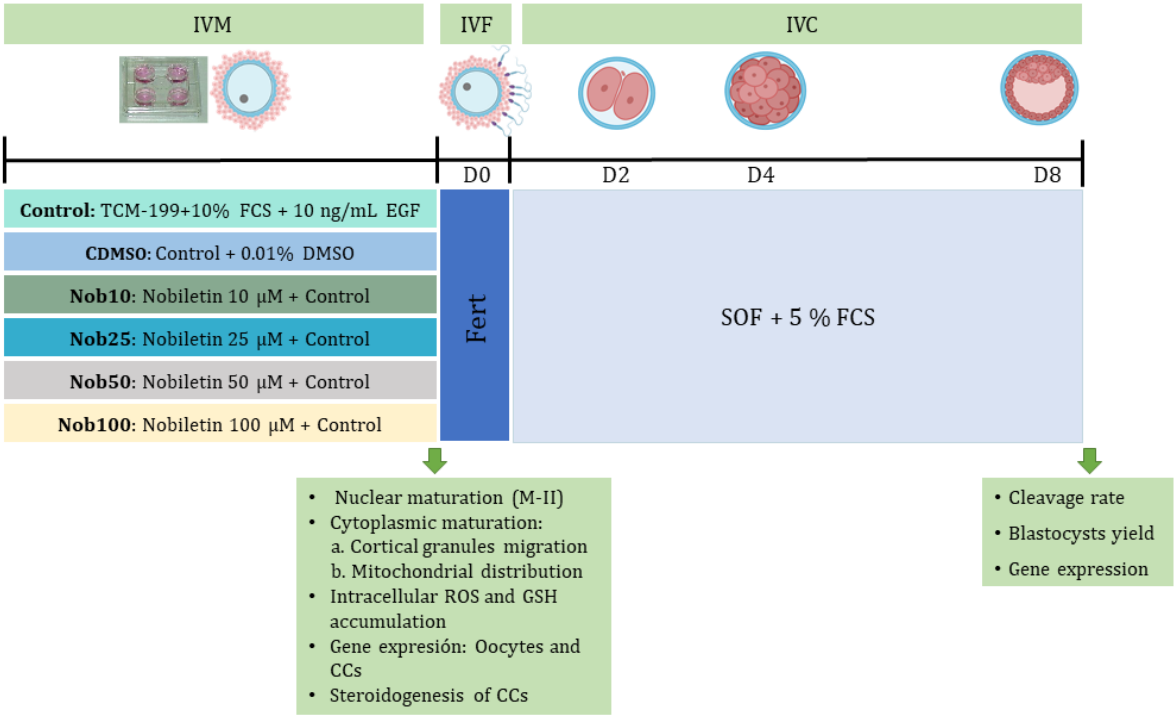

Figure 9. Experimental design of Experiment 1

3.5.2. Experiment 2: Development and quality in bovine embryos cultured *in vitro* with nobiletin at key periods of embryo development: minor (2 to 8-cell stage; MNEGA) or major (8 to 16-cell stage; MJEGA) phase of EGA

Because there are no data in the literature regarding the use of nobiletin during bovine IVC, it was necessary to perform preliminary experiments to determine the optimum concentration of nobiletin and corroborate if the same concentrations used in maturation media could be effective in the supplementation of embryo culture media.

3.5.2.1. Experiment 2a: Effect of nobiletin on early embryo development *in vitro*

In this experiment, the effect of nobiletin supplementation on embryo development during two developmental periods: (a) MNEGA: from 2-cell to 8-cell stage; and (b) MJEGA: from 8-cell to 16-cell stage; was determined by evaluating the cleavage rate at 54 hpi and blastocysts yield at Days 7 and 8 (Figure 10).

For this purpose, presumptive zygotes/embryos from 2- to 8- cell stage (MNEGA: 21-54 hpi) or embryos from 8- to 16-cell stage (MJEGA: 54 - 96 hpi) were cultured in SOF + 5% FCS alone (Control: n=730 and 621 for MNEGA and MJEGA respectively) or supplemented with 5, 10 or 25 µM nobiletin (Nob5: n=757 and 518; Nob10: n=695 and 553; and Nob25: n=521 and 424 for

MNEGA and MJEGA respectively), or 0.03% DMSO (CDMSO: n=695 and 622 for MNEGA and MJEGA respectively). For MJEGA phase groups, embryo culture until 8-cell stage (21-54 hpi) was performed in SOF + 5% FCS. At 54 hpi (MNEGA - Control: n=388; Nob5: n=386; Nob10: n=352; Nob25: n=254; CDMSO: n=368) or 96 hpi (MJEGA- Control: n=331; Nob5: n=315; Nob10: n=347; Nob25: n=210; CDMSO: n=331), embryos that reached the 8- or 16- cell stage, respectively, were transferred to SOF + 5% FCS and cultured until Day 8, maintaining the different experimental groups separately (Figure 10). Embryos were cultured in groups of 50 under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 ºC.

Considering that during the experiment it was necessary to preselect the embryos at different stages of development, the developmental parameters were calculated as follows: (I) developmental rate at 54 hpi: percentage of presumptive zygotes that developed to the 8-cell stage; (II) developmental rate at 96 hpi: percentage of selected 8-cell embryos at 54 hpi that developed to the 16-cell stage; and (III) blastocyst yield: percentage of selected 8-cell embryos (54 hpi) or 16-cell embryos (96 hpi) that continued in culture and developed to the blastocyst stage at Day 7 and Day 8. A representative number of 8-cell (MNEGA), 16-cell (MJEGA), and Day 7 blastocysts from both phases for each experimental group were frozen in liquid nitrogen (LN_2) in three groups of 10 and stored at - 80 ºC for gene expression.

Additionally, Day 7 blastocysts from both phases were either: (i) stained with MitoTracker DeepRed, Bodipy and Hoescht to evaluate mitochondrial activity, lipid content and total cell number, respectively (n≈30 per group); (ii) fixed in 4% PF for immunolocalization of p-AKT (n=10 per group); or (iii) frozen in LN_2 and stored at - 80 ºC for western blotting analysis of total and p-AKT (n=60 per group).

Twelve and ten replicates for MNEGA and MJEGA phases, respectively, were performed under the same assay conditions.

Only the experimental groups that showed higher blastocyst yield in this experiment (Nob5 and Nob10) in comparison with both control groups (Control and CDMSO) were used for experiment 2b and 2c.

3.5.2.2. Experiment 2b: Effect of nobiletin on the quality of *in vitro* produced blastocysts

To evaluate blastocyst quality of embryos produced *in vitro* with or without nobiletin supplementation during MNEGA or MJEGA, a representative number of Day 7 blastocysts (n≈30 per group/Experiment 2a) were stained with MitoTracker DeepRed, Bodipy and Hoescht to evaluate mitochondrial activity (intensity recorded in arbitrary units (a.u)), lipid content (lipid droplet area in μm^2) and total cell number, respectively. Blastocysts were examined using a laser-scanning confocal microscope or epifluorescence microscope and images obtained were evaluated using the ImageJ program.

To evaluate if nobiletin induces changes in the expression levels of genes related to embryo development and quality, three independent pools of 10 embryos per stage (8-cell, 16-cell, and blastocyst) obtained from each experimental group cultured with or without nobiletin during MNEGA or MJEGA (Experiment 2a), were used for gene expression analysis by qRT-PCR according to the procedures described above.

The selected genes have been linked to embryonic development and are essential in cell proliferation, differentiation, and embryo quality, such as PPARG coactivator 1 alpha (*PPARGC1A*); Peroxisome Proliferator-Activated Receptor Alpha (*PPARα*); Ribosomal Protein S6 Kinase Beta-1 (*RPS6KB1*); Cyclin Dependent Kinase 2 (*CDK2*); H3 Histone Family Member 3B (*H3-3B*) and H3 Histone Family Member 3A (*H3-3A*), including Nuclear Factor Erythroid 2-Like 2 (*NFE2L2*) and Glutathione Peroxidase 1 (*GPX1*) related with oxidative stress.

3.5.2.3. Experiment 2c: Nobiletin effect on the AKT pathway in blastocysts produced *in vitro*

To assess if nobiletin can interact with AKT pathway during *in vitro* embryo development, Day 7 blastocysts (n=10 - Experiment 2a) from each group were stained with p-AKT (Thr308/Ser473) for immunolocalization. To evaluate the phosphorylation level of AKT (Thr308 and Ser473), Day 7 blastocyst (n=30 - Experiment 2a) from each group were frozen in LN_2 for western blot analysis.

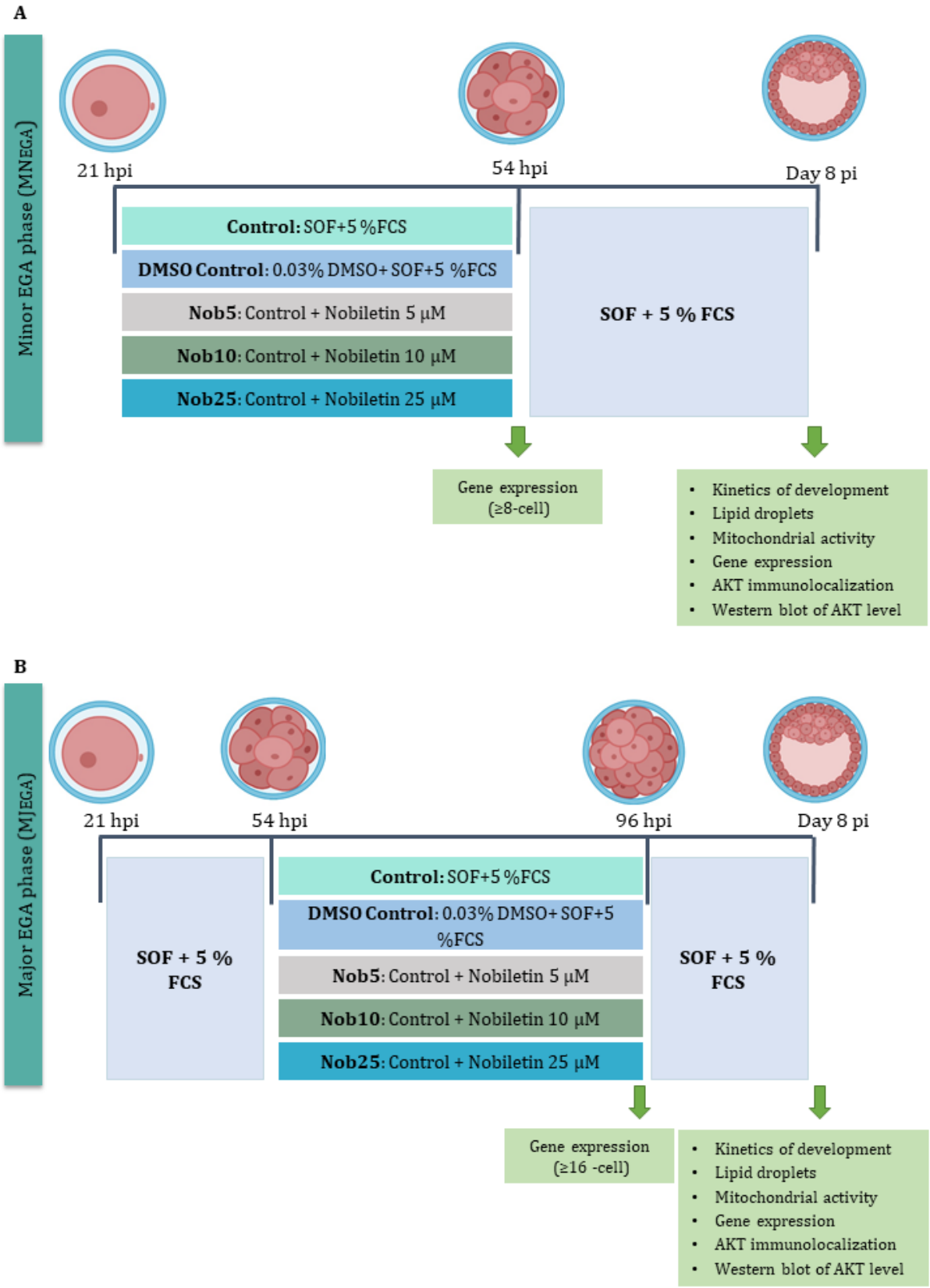

Figure 10. Experimental design of Experiment 2.

3.5.3. Experiment 3: Analysis of functional role of AKT signaling pathway by use of AKT inhibitors III or IV in bovine embryos and relationship between AKT signaling and nobiletin at key periods of bovine embryo development: MNEGA (2 to 8-cell stage) or MJEGA (8 to 16-cell stage) phase of EGA.

Considering there are few studies regarding the use of inhibitors of AKT pathway in bovine embryos, it was necessary to perform a preliminary experiment to determine the optimum concentration of AKT inhibitors during embryo culture. This experiment was also carried out during two phases of EGA (MNEGA and MJEGA) to determine if AKT inhibitor III (ALX-270-350 - Enzo Life Sciences, Farmingdale, NY), or AKT inhibitor IV (Sigma, 124,011-1MG), had a deleterious effect on early embryonic development and if nobiletin can counteracts these inhibitory effects.

Preliminary Experiment: Establishment of optimal AKT inhibitors III and IV concentration to prevent early embryo development *in vitro*

The optimal concentration of AKT inhibitors in *in vitro* culture media, under our experimental conditions, preventing early embryo development was established based on previous studies (Aparicio *et al.,* 2010; Ashry *et al.,* 2018).

In this preliminary experiment, presumptive zygotes from two individual replicates were cultured in SOF + 5% FCS alone (Control: n=100 for Inh III and n=100 for Inh IV) or supplemented with 75 µM (n=87), 50 µM (n=95) and 25 µM (n=91) AKT Inhibitor III (Inh III) or 3.5 µM (n=99), 2.5 µM (n=95) and 1.5 µM (n=95) AKT Inhibitor IV (Inh IV), or 0.03% DMSO (CDMSO: n=98 for Inh III and n=99 for Inh IV) until Day 8. Embryos were cultured in groups of 50 under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 ºC.

The developmental parameters were calculated as follows: (I) developmental rate at 54 hpi: percentage of presumptive zygotes that developed to the 8-cell stage; (II) developmental rate at 96 hpi: percentage of selected 8-cell embryos at 54 hpi that developed to the 16-cell stage; and (III) blastocyst yield: percentage of selected 8-cell embryos (54 hpi) or 16-cell embryos (96 hpi) that continued in culture and developed to the blastocyst stage at Day 7 and 8.

For Inh III, the development at 54 hpi (≥ 8-cell stage), 96 hpi (≥ 16-cell stage) and Day 8 (blastocyst yield) for all concentrations used was lethal (0.0%), while in both controls was the expected (≈64.5%; ≈69.0%; and ≈41.5%, respectively). For Inh IV none of the concentrations used showed any effect either at 54 hpi (range of ≥ 8-cell stage: 64.2 - 66.7%), 96 hpi (range of ≥ 16-

cell stage embryos: 65.1 - 70.5%) or Day 8 (range of blastocyst yield: 41.3 - 41.9%) being similar to both controls (≈64.5%; ≈70.0%; and ≈41.0%, respectively).

Concerning the above, in a following up experiment we had either to decrease the concentration of Inh III or increase the concentration of Inh IV. Therefore, presumptive zygotes from three individual replicates were cultured in SOF + 5% FCS alone (Control: n=151 for Inh III and n=153 for Inh IV) or supplemented with 15 µM (n=174), 7.5 µM (n=154) and 3.5 µM (n=153) Inh III or 10 µM (n=152), 7.0 µM (n=124) and 5 µM (n=138) Inh IV, or 0.03% DMSO (CDMSO: n=149 for Inh III and n=152 for Inh IV) until Day 8. Embryos were cultured in groups of 50 under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 ºC.

The development at 54 hpi (≥ 8-cell stage), 96 hpi (≥ 16-cell stage) and Day 8 (blastocyst yield) was reduced with 15 µM Inh III (55.2%; 40.6%; and 20.5% respectively) compared to 7.5 µM Inh III (62.3%; 58.3%; and 35.7%, respectively), 3.5 µM (62.1%; 65.3%; and 40.3%, respectively), Control (64.9%; 69.4%; and 41.2%, respectively) and CDMSO (65.8%; 70.4%; and 40.6%, respectively). Similarly, the development at 54 hpi (≥ 8-cell stage), 96 hpi (≥ 16-cell stage) and Day 8 (blastocyst yield) was reduced with 10 µM Inh IV (55.3%; 45.2%; and 18.4% respectively) compared to 7.0 µM Inh IV (63.7%; 64.6%; and 35.3%, respectively), 5 µM (65.2%; 65.6%; and 37.3%, respectively), Control (66.7%; 70.6%; and 40.3%, respectively) and CDMSO (66.4%; 69.3%; and 40.0%, respectively).

Consequently, concentrations of 15 µM Inh III and 10 µM Inh IV were used in the following experiments to inhibit AKT activity in bovine embryos *in vitro*.

3.5.3.1. Experiment 3a: Effect of AKT inhibitors III and IV on early embryo development and their AKT phosphorylation

AKT inhibitor III, known as SH6, is a cell-permeable reversible substrate competitive phosphatidylinositol analog that prevents the generation of PIP3 by PI3K which is required for AKT phosphorylation. AKT inhibitor IV is a cell-permeable reversible benzimidazole compound that inhibits AKT phosphorylation/activation by targeting the ATP binding site of a kinase upstream of AKT, but downstream of PI3K (Bhutani *et al.,* 2013). The effects of AKT inhibitor III or IV supplementation during two key developmental periods of EGA (MNEGA and MJEGA) were analyzed by evaluating the kinetics of embryo development at 54 hpi, 96 hpi and Days 7 and 8 (Figure 11).

In this experiment, presumptive zygotes/embryos from 2- to 8- cell stage (MNEGA: 21-54 hpi) or embryos from 8- to 16-cell stage (MJEGA: 54 - 96 hpi) were cultured in SOF + 5% FCS alone

(Control: n=212 and 210 for MNEGA and MJEGA respectively) or supplemented with 15 µM Inh III (n=293 and 277 for MNEGA and MJEGA respectively) or 10 µM Inh IV (n=317 and 273 for MNEGA and MJEGA respectively), or 0.03% DMSO (CDMSO: n=216 and 220 for MNEGA and MJEGA respectively). At 54 hpi (Control: n=99; Inh III: n=76; Inh IV: n=68; CDMSO: n=95) or 96 hpi (Control: n=60; Inh III: n=61; Inh IV: n=48; CDMSO: n=63), embryos that reached the 8- or 16- cell stage, respectively, were transferred to SOF + 5% FCS and cultured until Day 8, maintaining the different experimental groups separately (Figure 11). Embryos were cultured in groups of 50 under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 ºC.

The developmental parameters were calculated as in preliminary experiments: (I) developmental rate at 54 hpi: percentage of presumptive zygotes that developed to the 8-cell stage; (II) developmental rate at 96 hpi: percentage of selected 8-cell embryos at 54 hpi that developed to the 16-cell stage; and (III) blastocyst yield: percentage of selected 8-cell embryos (54 hpi) or 16-cell embryos (96 hpi) that continued in culture and developed to the blastocyst stage at Day 7 and 8.

To measure the effect of AKT inhibitors III and IV treatment on AKT signaling activity in bovine embryo, AKT-Thr308 phosphorylation levels were analyzed. For that, a representative number of 8-cell (MNEGA), 16-cell (MJEGA) (n=15/group/phase), and Day 7 blastocysts (n=10/group) from both phases for each experimental group were used for immunofluorescence analysis of phosphorylated pAKT.

Four replicates for each EGA phase were performed under the same assay conditions.

3.5.3.2. Experiment 3b: Effects of nobiletin supplementation on the developmental capacity of AKT inhibitor treated bovine embryos

In a previous study we established that 10 µM of nobiletin supplemented in culture media during MNEGA or MJEGA was beneficial to embryo development and to the quality of the produced blastocysts (Cajas *et al.,* 2019). Hence, in this experiment we investigated whether 10 µM of nobiletin supplementation to the *in vitro* culture media during MNEGA or MJEGA could overcome the detrimental effects of AKT cascade inhibition (Inh III and Inh IV) on development and quality of bovine embryos.

For this experiment, presumptive zygotes/embryos from 2- to 8- cell stage (MNEGA: 21-54 hpi) or embryos from 8- to 16-cell stage (MJEGA: 54 - 96 hpi) were cultured in SOF + 5% FCS alone (Control: n=560 and 594 for MNEGA and MJEGA respectively) or supplemented either with 15 µM AKT Inhibitor III (Inh III); 10 µM AKT Inhibitor IV (Inh IV) (Inh III: n=818 and 1080; Inh

IV: n=948 and 1109 for MNEGA and MJEGA respectively); 10 μM nobiletin (Nob: n=660 and 639 for MNEGA and MJEGA respectively); Nob with AKT Inhibitor III (Nob+Inh III: n=549 and 821 for MNEGA and MJEGA respectively); Nob with AKT Inhibitor IV (Nob+Inh IV: n=651 and 849 for MNEGA and MJEGA respectively) or 0.03% DMSO (CDMSO: n=597 and 651 for MNEGA and MJEGA respectively). For MJEGA phase groups, embryo culture until 8-cell stage (21-54 hpi) was performed in SOF + 5% FCS. At 54 hpi (MNEGA - Control: n=224; Inh III: n=190; Inh IV: n=174; Nob: n=317; Nob+Inh III: n=205; Nob+Inh IV: n=181; CDMSO: n=243) or 96 hpi (MJEGA- Control: n=161; Inh III: n=173; Inh IV: n=134; Nob: n=182; Nob+Inh III: n=184; Nob+Inh IV: n=205; CDMSO: n=171), embryos that reached the 8- or 16- cell stage, respectively, were transferred to SOF + 5% FCS and cultured until Day 8, maintaining the different experimental groups separately (Figure 11). Embryos were cultured in groups of 30 under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 ºC.

The developmental parameters were calculated as in previous experiments: (I) developmental rate at 54 hpi: percentage of presumptive zygotes that developed to the 8-cell stage; (II) developmental rate at 96 hpi: percentage of selected 8-cell embryos at 54 hpi that developed to the 16-cell stage; and (III) blastocyst yield: percentage of selected 8-cell embryos (54 hpi) or 16-cell embryos (96 hpi) that continued in culture and developed to the blastocyst stage at Day 7 and 8.

A representative number of 8-cell (MNEGA) and 16-cell embryos (MJEGA) from each experimental group were frozen in liquid nitrogen (LN_2) and stored at - 80 ºC, for gene expression analysis (three groups of 10: n=30 per group) and for western blot analysis of rpS6 (n=90 per group). Additionally, Day 7 blastocysts from both phases and experimental groups (n≈30 per group) were stained with anti-CDX2 and Hoescht to evaluate the total, TE and ICM cells.

Ten replicates for each EGA phase were performed under the same assay conditions.

3.5.3.3. Experiment 3c: Effects of nobiletin supplementation on the quality of AKT inhibit treated bovine embryos

To evaluate if nobiletin could compensate the negative effect of AKT inhibition changes in the expression levels of genes related to embryo development and quality, three independent pools of 10 embryos from 8-cell (MNEGA) and 16-cell stage (MJEGA) from all experimental groups (Experiment 3b), were frozen in LN_2 and stored at - 80 °C for analysis.

The selected genes have been linked to embryonic development and are essential in cell proliferation, differentiation and embryo quality, such as Caudal Type Homeobox 2 (*CDX2*),

Chromodomain helicase DNA-binding protein 1 (*CHD1*), DNA (Cytosine-5-)-Methyltransferase 3 Beta (*DNMT3B*), Fos-like antigen 1 (*FOSL1*), Krüppel-like factor 4 (*KLF4*), POU class 5 homeobox 1 (Oct3/4) (*POU5F1*), TEA Domain Transcription Factor 4 (*TEAD4*), including Nuclear Factor Erythroid 2-Like 2 (*NFE2L2*) and Glutathione Peroxidase 1 (*GPX1)*.

To evaluate if nobiletin could modulate the AKT inhibition on blastocyst cell number (TE and ICM), a representative number of Day 7 blastocysts from each group were incubated with anti-CDX2 antibody and then stained with Hoechst (n≈30 per group/Experiment 3b). Blastocysts were examined using a confocal microscope and images obtained were evaluated using the ImageJ program.

3.5.3.4. Experiment 3d: Effect of nobiletin supplementation on the activity of AKT signaling pathway in AKT inhibit treated early bovine embryos

In a previous experiment, we demonstrated that 10 μM of nobiletin supplementation in IVC during MNEGA or MJEGA increase AKT phosphorylation level on Day 7 blastocyst. Therefore, to elucidate the activation of this pathway downstream through AKT phosphorylation, the protein levels of rpS6 (phospho Ser235/236) was measured by Western blot in 8-cell (MNEGA) and 16-cell embryos (MJEGA) previously frozen in LN_2 and stored at -80 ºC (n=90 embryos/group/phase - Experiment 3b).

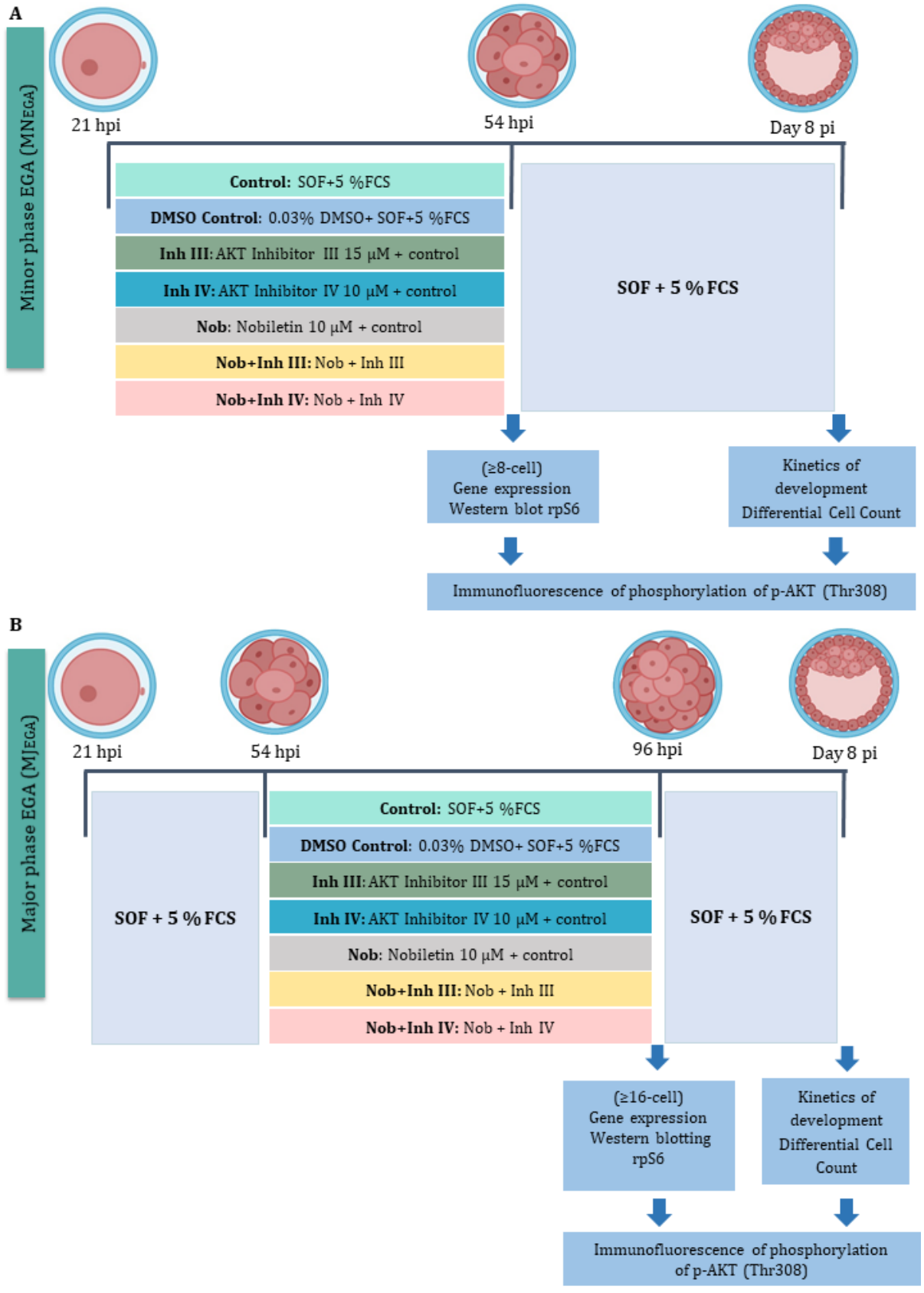

Figure 11. Experimental design of Experiment 3.

RESULTS

Experiment 1

Does nobiletin have a positive effect on in vitro maturation of bovine oocytes?

4. Results

4.1. Experiment 1: Nobiletin enhances oocyte maturation and subsequent embryo development and quality

4.1.1. Nobiletin enhances oocyte *in vitro* maturation and reduces oxidative stress

When evaluating the effect of nobiletin on nuclear maturation, we observed that a concentration of 25 (87.0 ± 0.6%) and 50 μM (89.3 ± 0.4%) increased (P<0.05) the percentage of oocytes reaching M-II compared to all other groups (Nob10: 72.9 ± 0.4%; Nob100: 71.5 ± 0.8%; Control: 71.7 ± 0.8%; and CDMSO: 70.5 ± 0.5%) (Figure 12, Table 3).

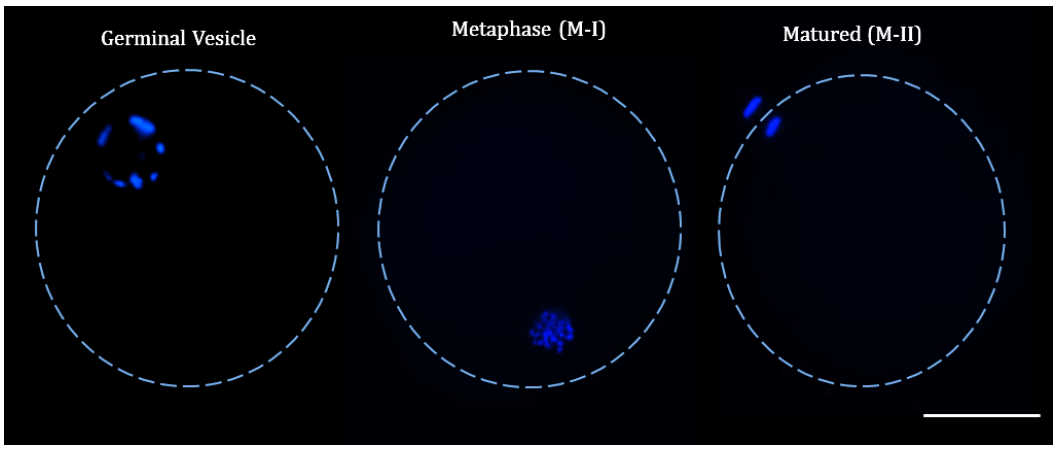

Figure 12. Representative images of nuclear maturation in bovine oocytes after *in vitro* maturation in the presence of nobiletin. Scale bar 50 μm.

The migration of CG to the cortical region of the oocyte, as well as mitochondrial distribution and their activity, were used as indicators to analyze cytoplasmic maturation. In the assessment of the CG distribution patterns oocytes matured in the presence of Nob25 (85.7 ± 0.3%) and Nob50 (89.9 ± 2.2%) displayed a higher incidence of migrated CG than oocytes in the Control (69.1 ± 1.1%), CDMSO (69.6 ± 0.9%), Nob10 (72.1 ± 1.0%) and Nob100 (71.2 ± 0.7%) groups (P<0.05). The presence of oocytes with a partially migrated pattern was lower (P<0.05) in Nob25 and Nob50 than all other groups. Similarly, the non-migrated pattern distribution of CG was lower (P<0.05) for nobiletin groups compared to the Control group, while for CDMSO, Nob10 and Nob100 no differences were observed (Table 3). Representative images of CG distribution in matured oocytes are presented in Figure 13.

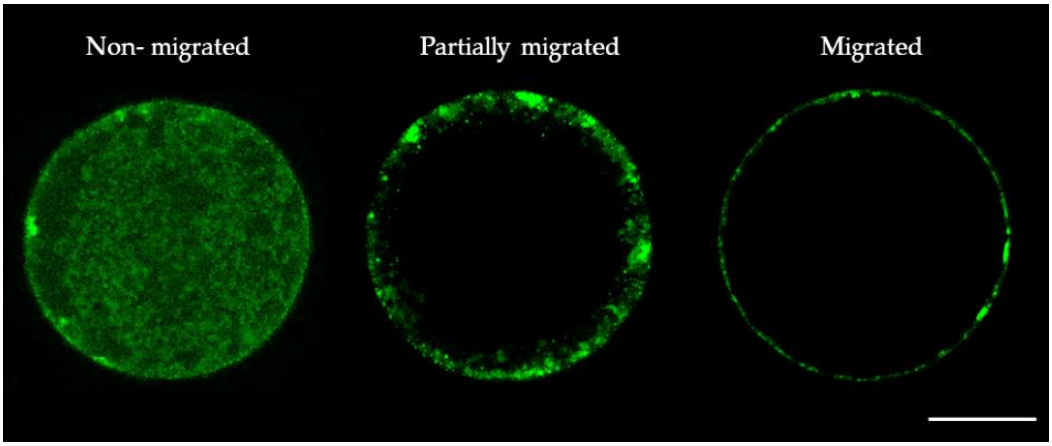

Figure 13. Representative fluorescent images of CG distribution patterns in bovine oocytes after *in vitro* maturation in the presence of nobiletin. Scale bar 50 µm.

Regarding the mitochondrial distribution patterns, we found higher migration (P<0.05) in oocytes matured with Nob25 (86.7 ± 0.6%) and Nob50 (88.9 ± 1.2%) compared to Control (71.3 ± 1.5%), CDMSO (69.7 ± 1.0%); Nob10 (73.7 ± 1.0%) and Nob100 (71.6 ± 0.5%) groups. The partially migrated mitochondrial pattern was lower (P<0.05) in the oocytes matured with Nob25 and Nob50 compared to all other groups, while the incidence of non-migrated mitochondria pattern was lower (P<0.05) only for Nob50 group (Figure 15, Table 3).

Quantification of mitochondrial activity in oocytes was measured by fluorescence intensity and a significant increase in intensity was observed in oocytes maturated with Nob25 and Nob50 compared to all other groups (P<0.05; Figure 14).

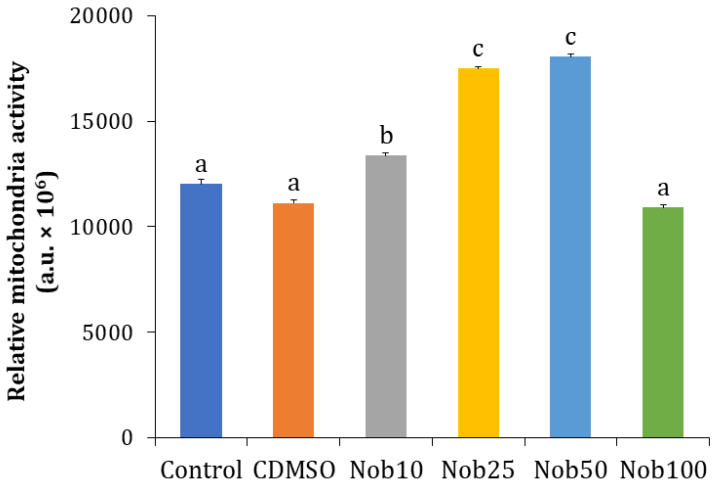

Figure 14. Quantification of relative mitochondrial activity.

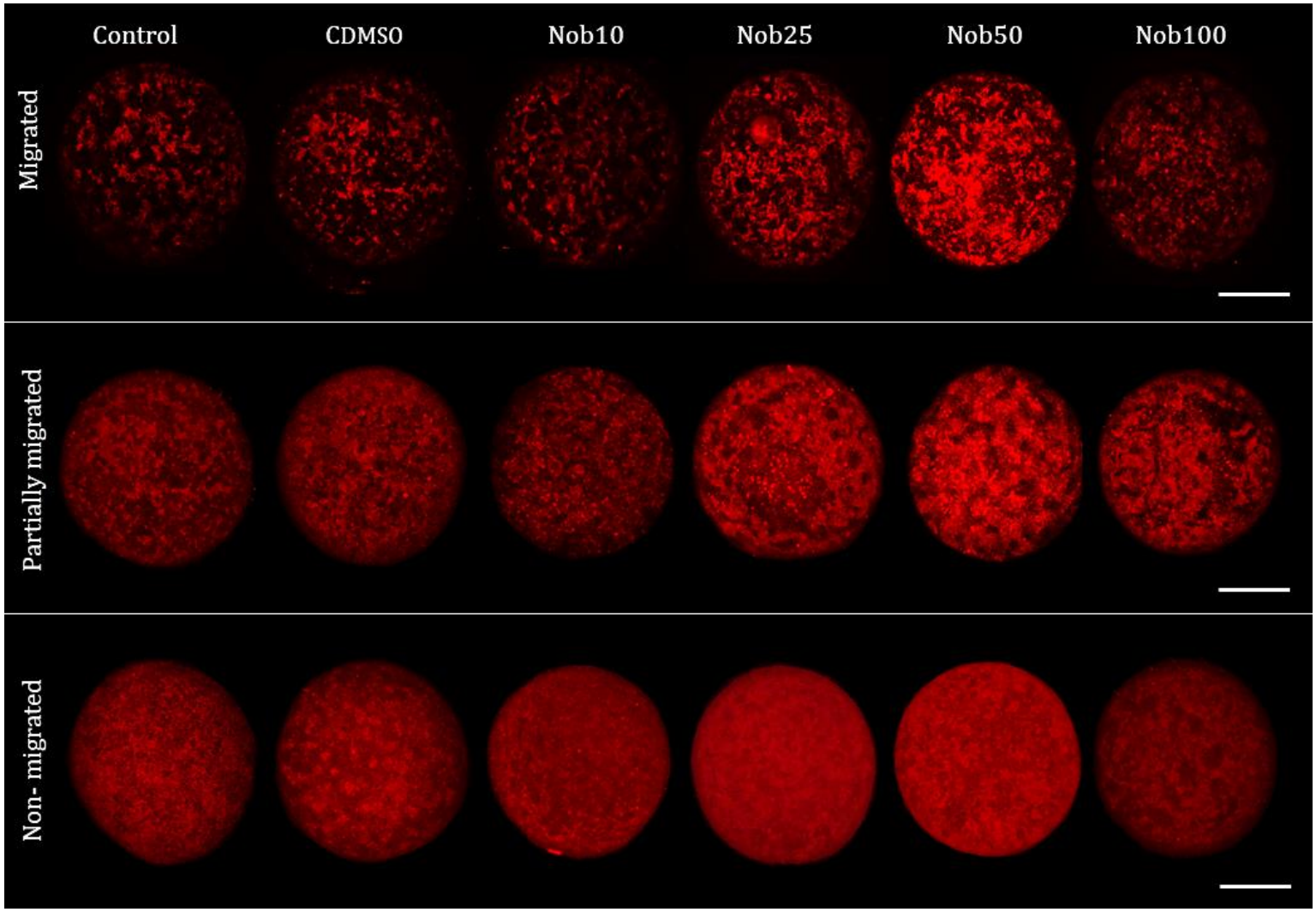

Figure 15. Representative fluorescent images of mitochondria migration pattern in bovine oocytes after *in vitro* maturation in the presence of nobiletin. Control: oocytes cultured in the presence of SOF and 5% FCS; CDMSO: oocytes cultured in the presence of SOF + 5% FCS supplemented with 0.01% DMSO; Nob10, Nob25, Nob50, Nob100 oocytes cultured in presence of SOF + 5% FCS supplemented with 10, 25, 50 and 100 μM nobiletin, respectively. Scale bar 50 μm.

Table 3. *In vitro* maturation of bovine oocytes in the presence of the nobiletin

	Control	**CDMSO**	**Nob10**	**Nob25**	**Nob50**	**Nob100**
Nuclear maturation n	117	122	133	146	149	144
Matured (M-II) n	84	86	97	127	133	103
(%±s.e.m)	(71.7 ± 0.8)^{a}	(70.5 ± 0.5)^{a}	(72.9 ± 0.4)^{a}	(87.0 ± 0.6)^{b}	(89.3 ± 0.4)^{b}	(71.5 ± 0.8)^{a}
Immature n	33	36	36	19	16	41
(%±s.e.m)	(28.2 ± 0.7)^{a}	(29.5 ± 0.5)^{a}	(27.1 ± 0.4)^{a}	(12.9 ± 0.6)^{b}	(10.7 ± 0.4)^{b}	(28.4 ± 0.8)^{a}
Cytoplasmic maturation						
Cortical granules distribution						
n	58	66	72	70	78	70
Migrated n	40	46	52	60	70	50
(%±s.e.m)	(69.1 ± 1.1)^{a}	(69.6 ± 0.9)^{a}	(72.1 ± 1.0)^{a}	(85.7 ± 0.3)^{b}	(89.9 ± 2.2)^{b}	(71.2 ± 0.7)^{a}
Partially migrated n	10	12	15	7	7	15
(%±s.e.m)	(17.2 ± 2.6)^{a}	(18.2 ± 1.7)^{a}	(20.9 ± 0.7)^{a}	(9.9 ± 1.6)^{b}	(8.8 ± 1.3)^{b}	(21.5 ± 0.6)^{a}
Non-migrated n	8	8	5	3	1	5
(%±s.e.m)	(13.7 ± 1.9)^{a}	(12.2 ± 2.0)^{ac}	(6.9 ± 0.2)^{bc}	(4.4 ± 1.8)^{b}	(1.2 ± 1.2)^{b}	(7.3 ± 0.2)^{bc}
Mitochondrial distribution						
n	59	56	61	76	71	74
Migrated n	42	39	45	66	63	53
(%±s.e.m)	(71.3 ± 1.5)^{a}	(69.7 ± 1.0)^{a}	(73.7 ± 1.0)^{a}	(86.7 ± 0.6)^{b}	(88.9 ± 1.2)^{b}	(71.6 ± 0.5)^{a}
Partially migrated n	10	11	11	5	7	13
(%±s.e.m)	(17.0 ± 0.5)^{a}	(19.6 ± 1.1)^{a}	(17.9 ± 1.0)^{a}	(6.7 ± 0.3)^{b}	(9.8 ± 1.5) ^{b}	(17.5 ± 1.5)^{a}
Non-migrated n	7	6	5	5	1	8
(%±s.e.m)	(11.7 ± 1.8)^{a}	(10.8 ± 1.5)^{a}	(8.3 ± 0.4)^{a}	(6.6 ± 0.3)^{ab}	(1.3 ± 1.3)^{b}	(10.8 ± 1.7)^{a}

n: number of oocytes assigned per group. Control: oocytes cultured in the presence of SOF and 5% FCS; CDMSO: oocytes cultured in the presence of SOF + 5% FCS supplemented with 0.01% DMSO; Nob10, Nob25, Nob50, Nob100 oocytes cultured in presence of SOF + 5% FCS supplemented with 10, 25, 50 and 100 μM nobiletin, respectively. Data are the mean ± s.e.m. Within columns, values with different superscript letters differ significantly (P<0.05).

When evaluating the effect of nobiletin on oxidative stress, through a relative of ROS and GSH fluorescence intensity in maturated oocytes, we observed that the intensity in both parameters was lower (P<0.05) in Nob25 and Nob50 groups compared with oocytes matured with Nob10 and Nob100 and control groups (Figure 16).

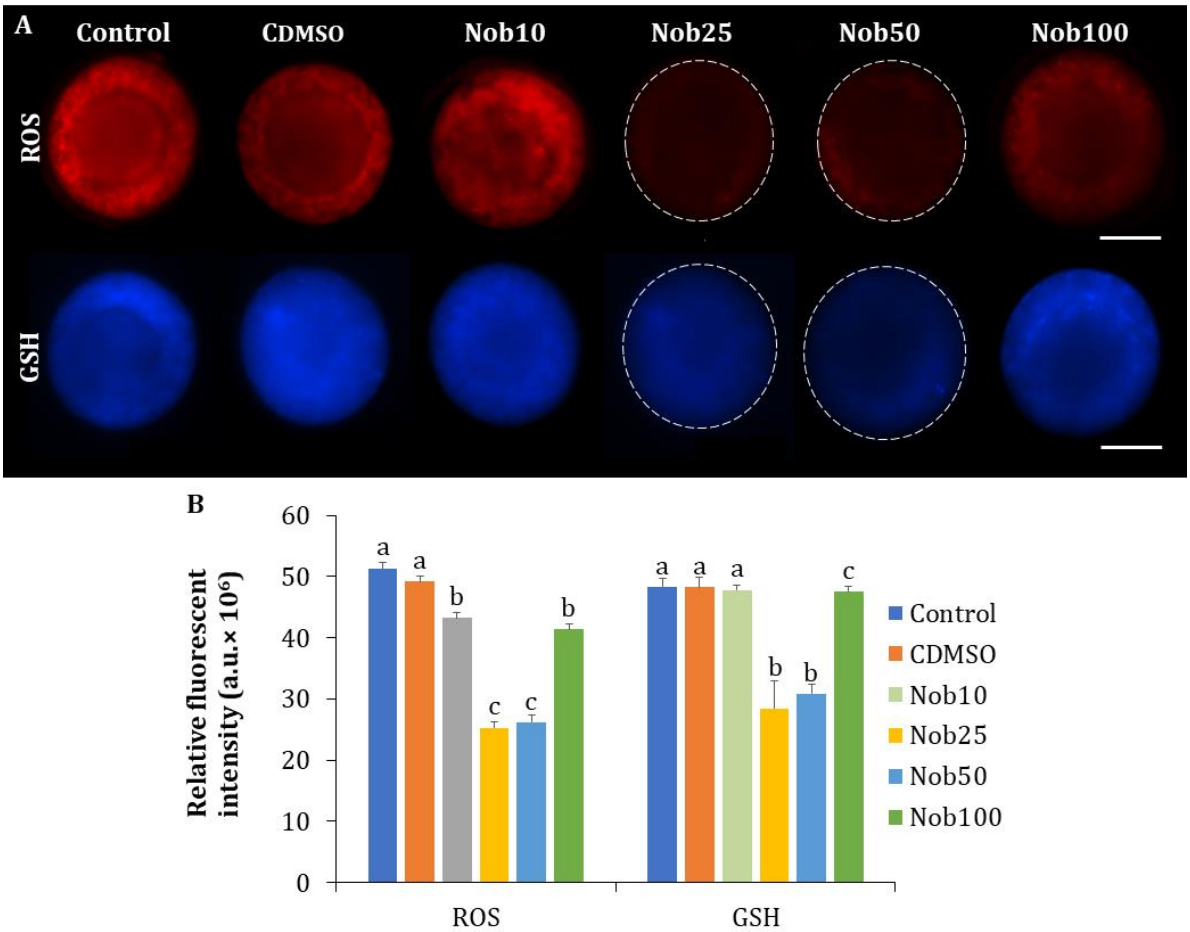

Figure 16. ROS and GSH fluorescence intensity in bovine oocytes after *in vitro* maturation in the presence of nobiletin (A) Representative fluorescent images of reactive oxygen species (ROS) and glutathione (GSH) fluorescence intensity in bovine oocytes after *in vitro* maturation in the presence of nobiletin. (B) Quantification of relative fluorescent intensity of ROS and GSH in bovine oocytes after *in vitro* maturation in the presence of nobiletin. Data are the mean ± s.e.m. Values with different superscript letters differ significantly (P<0.05). Scale bar 50 µm.

Based on these results and to verify the effects of nobiletin on *in vitro* maturation and oxidative stress we analyzed gene expression in oocytes and their CCs. Only the experimental groups that showed better qualitative parameters in the previous experiments (Nob25 and Nob50) were used in comparison with both control groups (Control and CDMSO). Supplementation of IVM medium with nobiletin, irrespective of the concentration, induced the upregulation of *MAPK1* and *BMP15* (developmental-related transcripts) and downregulation of *SOD2* and *CYP51A1* (oxidative stress transcripts) in oocytes after IVM when compared with

control groups (P<0.05). No significant differences were observed for the remaining transcripts studied (*BCL2*, *GAPDH*, *GDF9*) (Figure 17A). In CCs, nobiletin produced changes in the expression levels of genes related to quality and development (Figure 17B). *BMP15* (development) and *GJA1* (cell junctions) transcripts were upregulated (P<0.05), while the expression of the oxidative stress (*SOD2, CYP51A1*) and apoptosis (*BCL2*) genes were downregulated in nobiletin groups compared to controls (P<0.05). No significant differences were observed for the remaining transcripts studied (*ABCB1, CDH1, CLIC1, FOS, GAPDH, GDF9, IGF2R,* and *MAPK1*).

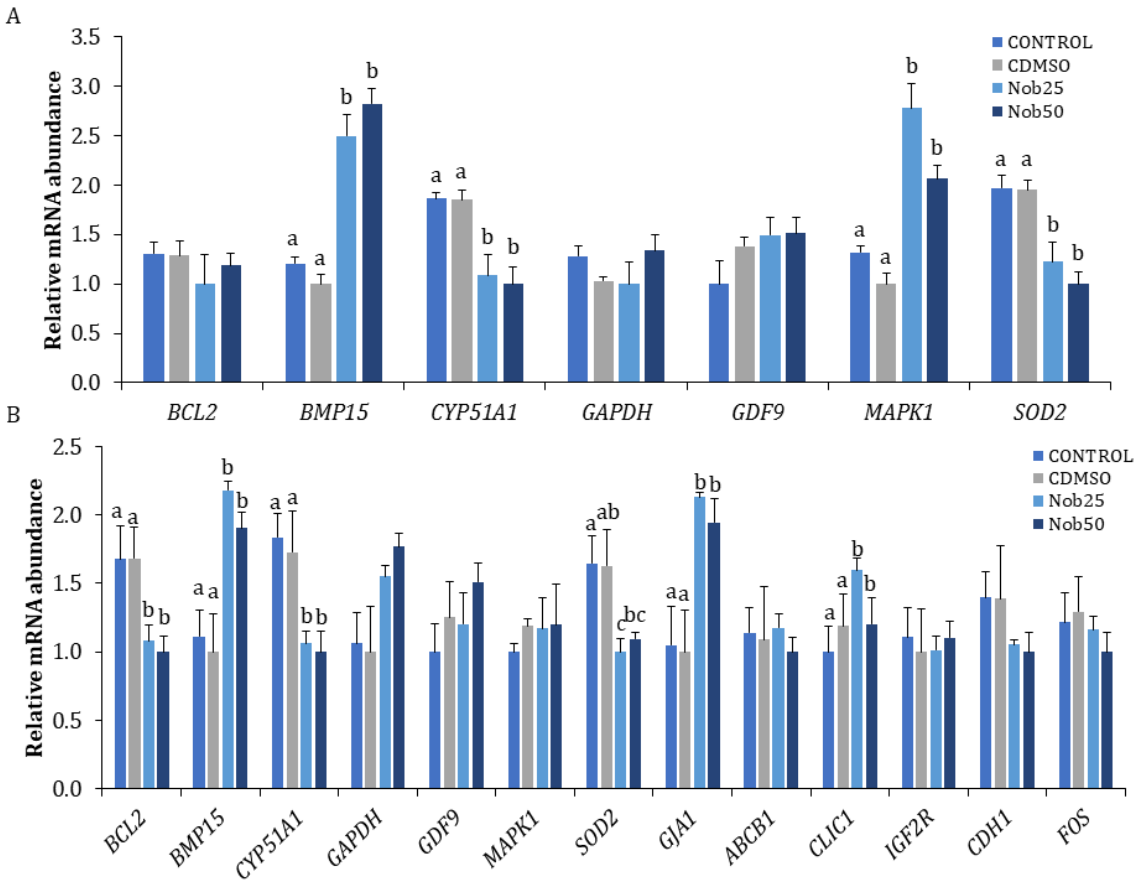

Figure 17. Relative mRNA transcript abundance (normalized against that of the endogenous control H2A histone family member Z (*H2AFZ*) gene and actin beta (*ACTB*)). (A) Bovine oocytes after *in vitro* maturation in the presence of nobiletin. (B) Bovine cumulus cells (CCs) after *in vitro* maturation in the presence of nobiletin. Data are the mean ± s.e.m. Different letters above columns indicate significant differences in gene expression among the experimental groups (P<0.05).

4.1.2. Nobiletin increases estradiol and progesterone production by cumulus cells

After IVM, a significant increase in E_2 production by CCs was found in maturation medium supplemented with Nob25 (368.6 ± 27.3 pg/mL) and Nob50 (421.0 ± 28.2 pg/mL) compared with the rest of the groups (Control: 233.2 ± 16.9 pg/mL; CDMSO: 212.4 ± 11.8 pg/mL; Nob10: 216.2 ± 20.0 pg/mL; and Nob100: 250.2 ± 24.4 pg/mL ($P<0.05$; Figure 18A). Likewise, a significant increase in P_4 production by CCs in media after maturation was detected within Nob25 (19.7 ± 0.3 ng/mL) and Nob50 (20.2 ± 0.2 ng/mL) groups compared with the remaining groups ($P<0.05$; Figure 18B).

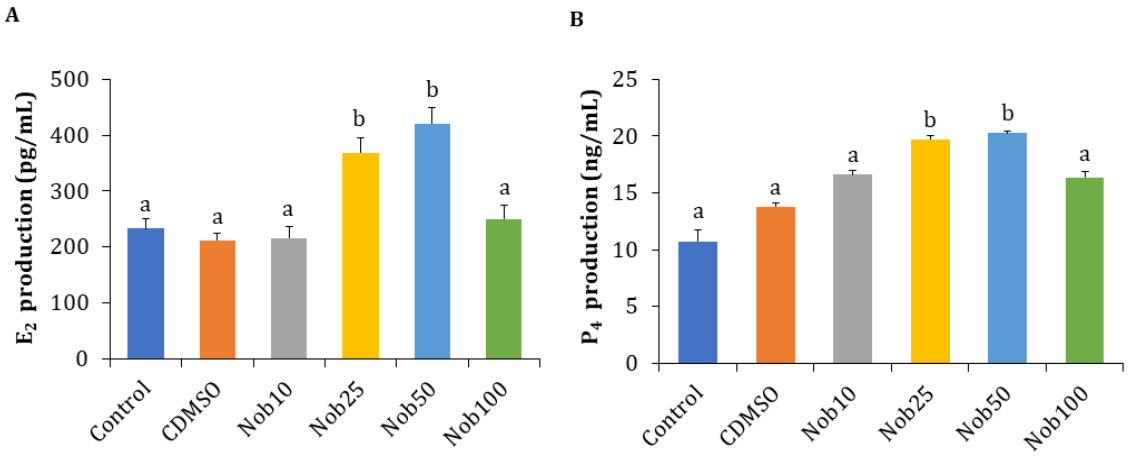

Figure 18. Steroidogenic production of CCs after *in vitro* maturation using different concentrations of nobiletin. (A) Steroidogenic production of Estradiol (E_2). (B) Steroidogenic production of Progesterone (P_4). Bars represent mean concentrations produced by CCs under each different experimental condition. Data are the mean ± s.e.m. Values with different superscript letters differ significantly ($P<0.05$).

4.1.3. Nobiletin increases embryo development and quality

Embryonic development was assessed after IVM in the presence of nobiletin (Table 4). Cleavage rate and cumulative blastocyst yield at Day 7 and 8 were higher ($P<0.05$) for Nob25 and Nob50 compared to all other groups. Based on these results, and for blastocysts quality evaluation only the Nob10 and Nob25 groups with both control groups (Control and CDMSO) were used for gene expression analysis.

The expression of *MAPK1* was upregulated, while *CLIC1* was downregulated in blastocysts produced after oocyte maturation with nobiletin supplementation, irrespective of the concentration, compared with blastocysts from control groups ($P<0.05$). The expression of *CYP51A1* was upregulated in blastocysts from the Nob50 group compared to blastocysts from

control groups (P<0.05). No significant differences were observed for the remaining transcripts studied (*ABCB1*, *BCL2*, *BMP7*, *GAPDH*, *GDF9*, *IGF2R*, and *SOD2*) (Figure 19).

Table 4. Effect of nobiletin on *in vitro* maturation of bovine oocytes and subsequent embryonic development

	Total no. Presumptive zygotes in culture	Cleavage rate	Blastocyst yield	
			Day 7	Day 8
		n (% ± s.e.m)	n (% ± s.e.m)	n (% ± s.e.m)
Control	359	267 (74.2 ± 0.4)^{a}	76 (21.1 ± 0.4)^{a}	92 (25.8 ± 0.5)^{a}
CDMSO	378	278 (73.6 ± 0.5)^{a}	78 (20.9 ± 0.4)^{a}	98 (26.1 ± 0.7)^{a}
Nob10	397	300 (75.6 ±0.3)^{a}	75 (18.9 ± 0.4)^{a}	90 (23.1 ± 0.7)^{a}
Nob25	372	335 (89.9 ± 0.4)^{b}	90 (24.4 ± 0.5)^{b}	119 (32.2 ± 0.8)^{b}
Nob50	336	307 (91.3 ± 0.3)^{b}	86 (25.7 ± 0.6)^{b}	117 (35.3 ± 0.8)^{b}
Nob100	414	306 (74.0 ± 0.6)^{a}	76 (18.9 ± 0.9)^{a}	100 (24.5 ± 1.0)^{a}

n: number of oocytes assigned per group. Control: blastocysts cultured in the presence of SOF and 5% FCS; CDMSO: blastocysts cultured in the presence of SOF + 5% FCS supplemented with 0.01% DMSO; Nob10, Nob25, Nob50, Nob100 oocytes cultured in presence of SOF + 5% FCS supplemented with 10, 25, 50 and 100 µM nobiletin, respectively. Data are the mean ± s.e.m. Within columns, values with different superscript letters differ significantly (P<0.05).

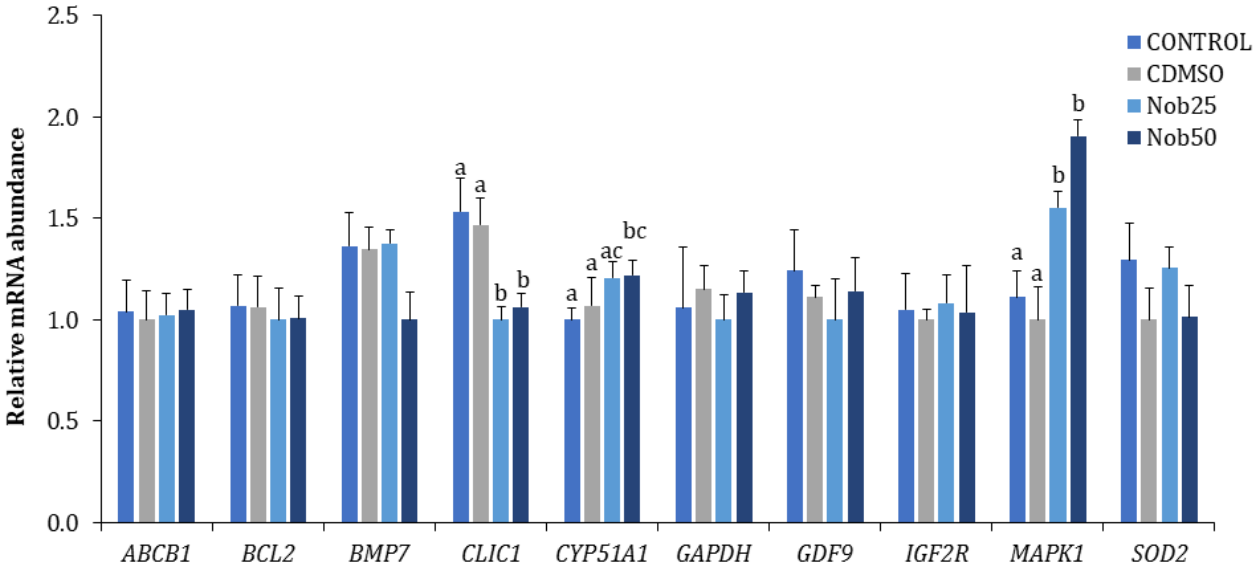

Figure 19. Relative mRNA transcript abundance (normalized against that of the endogenous control H2A histone family member Z (*H2AFZ*) gene and actin beta (*ACTB*)) of blastocysts D7 developed from oocytes matured in the presence of nobiletin. Data are the mean ± s.e.m. Different letters above columns indicate significant differences in gene expression among the experimental groups (P<0.05).

Experiment 2

Does nobiletin have a positive effect on in vitro culture during MNEGA or MJEGA?

4.2. Experiment 2: Nobiletin enhances the development and quality of bovine embryos *in vitro* during two key periods of embryonic genome activation

4.2.1. Nobiletin during MNEGA or MJEGA enhances early embryo development *in vitro*

For all experimental groups, only embryos that reached the 8-cell stage at 54 h post-insemination (hpi) were selected for the study. As shown in Figure 20A for MNEGA phase, no differences were observed in cleavage rate at 54 hpi, which ranged from 82.3 ± 1.0 to 85.5 ± 0.5%. At 54 hpi, no differences were observed either in the proportion of embryos that reached the 8-cell stage, which ranged from 57.1 ± 1.4 to 60.4 ± 0.7%. Consequently, a similar proportion of embryos with a delayed development (<8 cells), which ranged from 22.6 ± 0.9 to 26.6 ± 1.2%, was observed (Figure 20B). Blastocyst yield at Day 7 and Day 8 (Figure 20C) was significantly higher (P<0.001) for Nob5 (39.7 ± 0.8 and 42.7 ± 1.0%, respectively) and Nob10 (41.0 ± 1.0 and 44.4 ± 1.3%), compared to Control (32.7 ± 0.7 and 34.6 ± 0.7%); CDMSO (32.8 ± 0.5 and 34.9 ± 0.4%) and Nob25 (31.8 ± 1.7 and 34.6 ± 1.2%) (Table 5).

Table 5. Cleavage rate and kinetics of development at 54 h post-insemination and cumulative blastocyst rates on Days 7 and 8 after *in vitro* culture with nobiletin supplemented during MNEGA.

	IVC N	Total cleaved 54 hpi N (% ± s.e.m)	< 8 cells N (% ± s.e.m)	≥ 8 cells N (% ± s.e.m)	IVC 54 hpi N	Blastocysts	
						Day 7 N (% ± s.e.m)	Day 8 N (% ± s.e.m)
Control	730	602 (82.3 ± 1.0)	164 (22.6 ± 0.9)	438 (59.7 ± 1.1)	388	126 (32.7 ± 0.7)[a]	132 (34.6 ± 0.7)[a]
CDMSO	695	595 (85.5 ± 0.5)	177 (25.1 ± 0.8)	418 (60.4 ± 0.7)	368	120 (32.8 ± 0.5)[a]	128 (34.9 ± 0.4)[a]
Nob5	757	625 (82.4 ± 1.0)	189 (25.3 ± 0.8)	436 (57.1 ± 1.4)	386	151 (39.7 ± 0.8)[b]	163 (42.7 ± 1.0)[b]
Nob10	695	586 (84.5 ± 0.6)	184 (26.3 ± 0.8)	402 (58.2 ± 1.1)	352	144 (41.0 ± 1.0)[b]	157 (44.4 ± 1.3)[b]
Nob25	521	442 (85.0 ± 1.0)	138 (26.6 ± 1.2)	304 (58.5 ± 1.6)	254	81 (31.8 ± 1.7)[a]	89 (34.6 ± 1.2)[a]

MNEGA: embryos cultured from zygotes to 8-cell stage: 21–54 hpi in SOF+5% FCS (Control), supplemented or not with 5, 10 or 25 µM of nobiletin (Nob5, Nob10 or Nob25, respectively), or with 0.03% dimethyl sulfoxide (CDMSO). IVC - N: number of presumptive zygotes in culture. IVC - 54 hpi - N: number of 8-cell embryos in culture at 54 hpi. Blastocysts yield on D7 and D8 is calculated from the 8-cell embryos in culture at 54 hpi. Data are the mean ± s.e.m. Within columns, different superscript letters indicate significant difference (P<0.001) between treatments.

During MJEGA, cleavage rate at 54 hpi was 86.6 ± 0.2% and the proportion of embryos that reached the 8-cell stage was 71.1 ± 0.4% while the proportion of embryos with a delayed development (<8 cells) was 15.5 ± 0.3% (Figure 20D). At 96 hpi a significantly (P<0.001) higher proportion of embryos reached the 16-cell stage in Nob5 and Nob10 groups (70.1 ± 0.5% and 69.9 ± 0.4%, respectively) compared to Control (60.0 ± 0.4%), CDMSO (60.7 ± 0.4%) and Nob25 (60.8 ± 0.8%) groups (Figure 20E). As a consequence, a significantly lower proportion of embryos with a delayed development (<16 cells) was observed in Nob5 and Nob10 compared to the other groups (Nob5: 29.9 ± 0.5% and Nob10: 30.1 ± 0.4% vs Control: 40.0 ± 0.4%, CDMSO: 39.3 ± 0.4% and Nob25: 39.2 ± 0.8%, P<0.001). On Day 7 and Day 8, blastocyst yield was significantly higher (P<0.001) for Nob10 (54.5 ± 1.1% and 61.0 ± 0.8%, respectively) compared to Control (38.4 ± 1.1% and 47.3 ± 1.4%), CDMSO (35.8 ± 1.0% and 44.0 ± 1.1%), Nob5 (46.6 ± 0.8% and 52.5 ± 1.5%) and Nob25 (35.9 ± 1.5% - 42.5 ± 1.3%) groups, while Nob5 was higher (P<0.001) compared to Nob25 and both control groups (Figure 20F, Table 6).

Table 6. Cleavage rate and kinetics of development at 54 and 96 hpi and cumulative blastocyst rates on days 7 and 8 after *in vitro* culture with nobiletin supplemented during MJEGA.

	IVC N	Total cleaved 54 hpi N (% ± s.e.m)	< 8 cells N (% ± s.e.m)	≥ 8 cells N (% ± s.e.m)	Total cleaved 96 hpi N	< 16 cells N (% ± s.e.m)	≥ 16 cells N (% ± s.e.m)	IVC 96 hpi	Blastocysts	
									Day 7 N (% ± s.e.m)	Day 8 N (% ± s.e.m)
Control	867	754 (87.0 ± 0.5)	133 (15.6 ± 0.3)	621 (71.4 ± 0.7)	621	250 (40.0 ± 0.4)[a]	371 (60.0 ± 0.4)[a]	331	126 (38.4 ± 1.1)[a]	154 (47.3 ± 1.4)[a]
CDMSO	878	761 (86.8 ± 0.7)	139 (16.3 ± 0.5)	622 (70.5 ± 0.7)	622	246 (39.3 ± 0.4)[a]	376 (60.7 ± 0.4)[a]	331	120 (35.8 ± 1.0)[a]	146 (44.0 ± 1.1)[a]
Nob5	724	623 (86.4 ± 0.5)	105 (14.9 ± 0.9)	518 (71.5 ± 0.9)	518	153 (29.9 ± 0.5)[b]	365 (70.1 ± 0.5)[b]	315	148 (46.6 ± 0.8)[b]	169 (52.5 ± 1.5)[b]
Nob10	773	667 (86.3 ± 0.4)	114 (14.8 ± 0.6)	553 (71.5 ± 0.7)	553	166 (30.1 ± 0.4)[b]	387 (69.9 ± 0.4)[b]	347	190 (54.5 ± 1.1)[c]	210 (61.0 ± 0.8)[c]
Nob25	597	516 (86.2 ± 0.6)	92 (15.7 ± 0.9)	424 (70.5 ± 1.3)	424	166 (39.2 ± 0.8)[a]	259 (60.8 ± 0.8)[a]	210	74 (35.9 ± 1.5)[a]	88 (42.5 ± 1.3)[a]

MJEGA: embryos cultured from 8-cell to 16-cell stage: 54–96 hpi in SOF+5% FCS (Control), supplemented or not with 5, 10 and 25 µM of nobiletin (Nob5, Nob10 and Nob25, respectively), or with 0.03% dimethyl sulfoxide (CDMSO). IVC - N: number of presumptive zygotes in culture. IVC - 54 hpi - N: number of 8-cell embryos in culture at 54 hpi. IVC - 96 hpi - N: number of 16-cell embryos in culture at 96 hpi. Blastocysts yield on D7 and D8 is calculated from the 16-cell embryos in culture at 96 hpi. Data are the mean ± s.e.m. Within columns, different superscript letters indicate significant difference ($P<0.001$) between treatments.

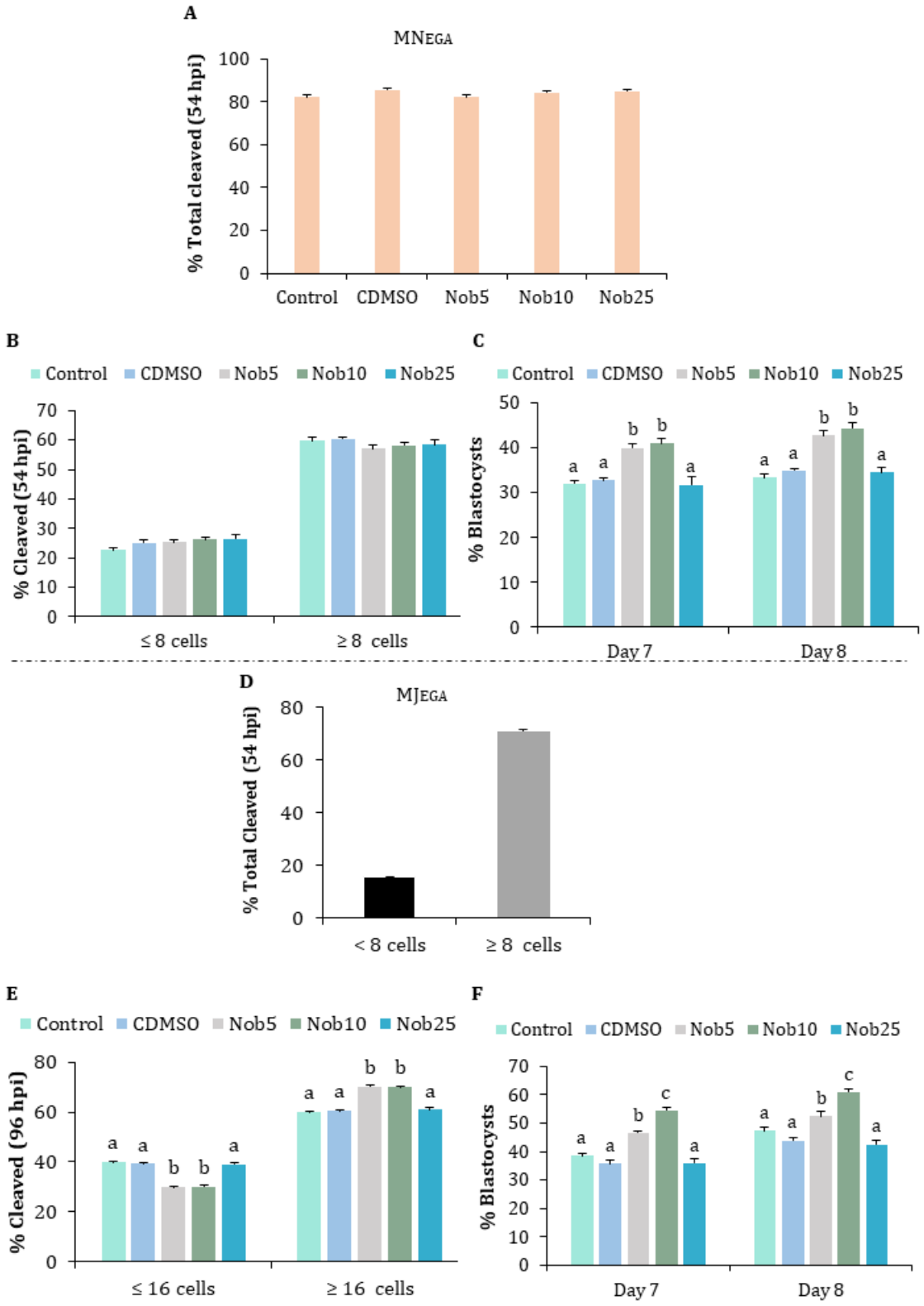

Figure 20. Developmental rates of *in vitro* produced bovine embryos cultured during 21 - 54 hpi (MNEGA: A, B, C) or during 54 - 96 hpi (MJEGA: D, E, F) with or without nobiletin. (A, D) Total cleavage rate at 54 hpi; (B, E) embryos ≥ or < 8-cell stage at 54 hpi and ≥ or < 16-cell stage at 96 hpi; (C, F) blastocyst rate on Days 7 and 8 pi (*in vitro* fertilization = Day 0), from embryos cultured in SOF+5% FCS (Control), supplemented or not with 5 (Nob5), 10 (Nob10) or 25 µM (Nob25) nobiletin or with 0.03% dimethyl sulfoxide (CDMSO) during MNEGA or MJEGA respectively. Results are expressed as mean ± s.e.m. Significant differences ($P<0.001$) are indicated with different letters.

4.2.2. Nobiletin during MNEGA or MJEGA increases the quality of *in vitro* produced blastocysts

Only the experimental groups that showed better blastocyst yield in the previous experiment (Nob5 and Nob10 during MNEGA or MJEGA) were used for embryo quality evaluation in comparison with both control groups (Control and CDMSO).

The mitochondrial activity was higher (P<0.001) in blastocysts from Nob5 and Nob10 groups, from either MNEGA or MJEGA phase, compared with both control groups (Figure 21).

When analyzing the lipid content, we observed that the total area of lipid droplets in blastocysts resulting from treatments during MNEGA or MJEGA was significantly reduced (P<0.001) in Nob5 and Nob10 groups compared with the control groups (Figure 22).

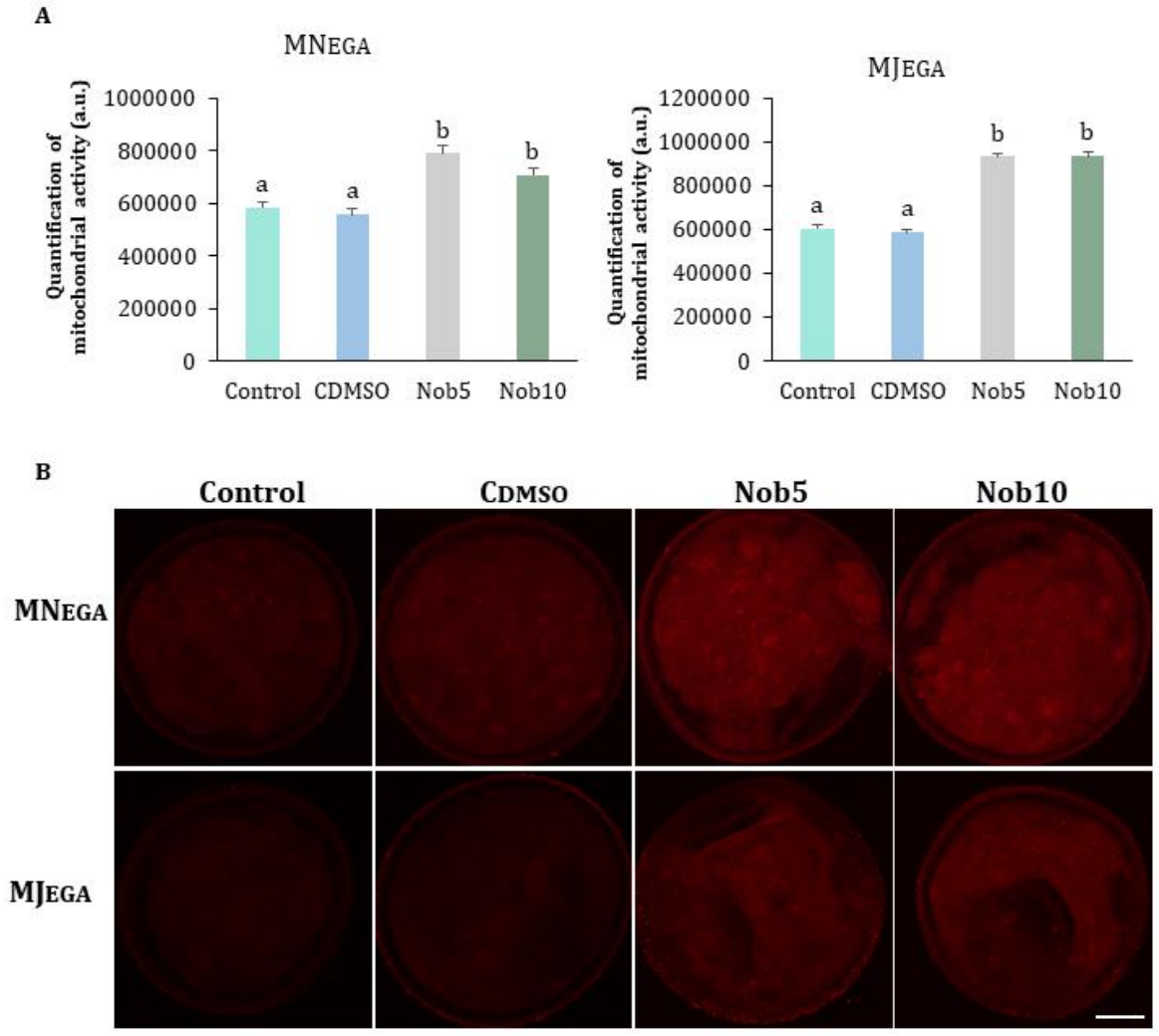

Figure 21. Mitochondrial activity in *in vitro* produced bovine blastocysts cultured during 21 - 54 hpi (MNEGA: presumptive zygote to 8-cell stage) or during 54 - 96 hpi (MJEGA: 8- to 16-cell stage) with or without nobiletin. (A) Quantification of mitochondrial fluorescence intensity in arbitrary units (a.u) in Day 7 blastocysts cultured in SOF+5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (CDMSO) during MNEGA or MJEGA phase. Data are the

mean±s.e.m. Significant differences (P<0.001) are indicated with different letters. (B) Representative fluorescence images of mitochondrial activity in Day 7 blastocysts from all experimental groups (Control, Nob5 Nob10 CDMSO) in both phases (MNEGA or MJEGA). Images were captured on 63× objective. Scale bar 50 µm.

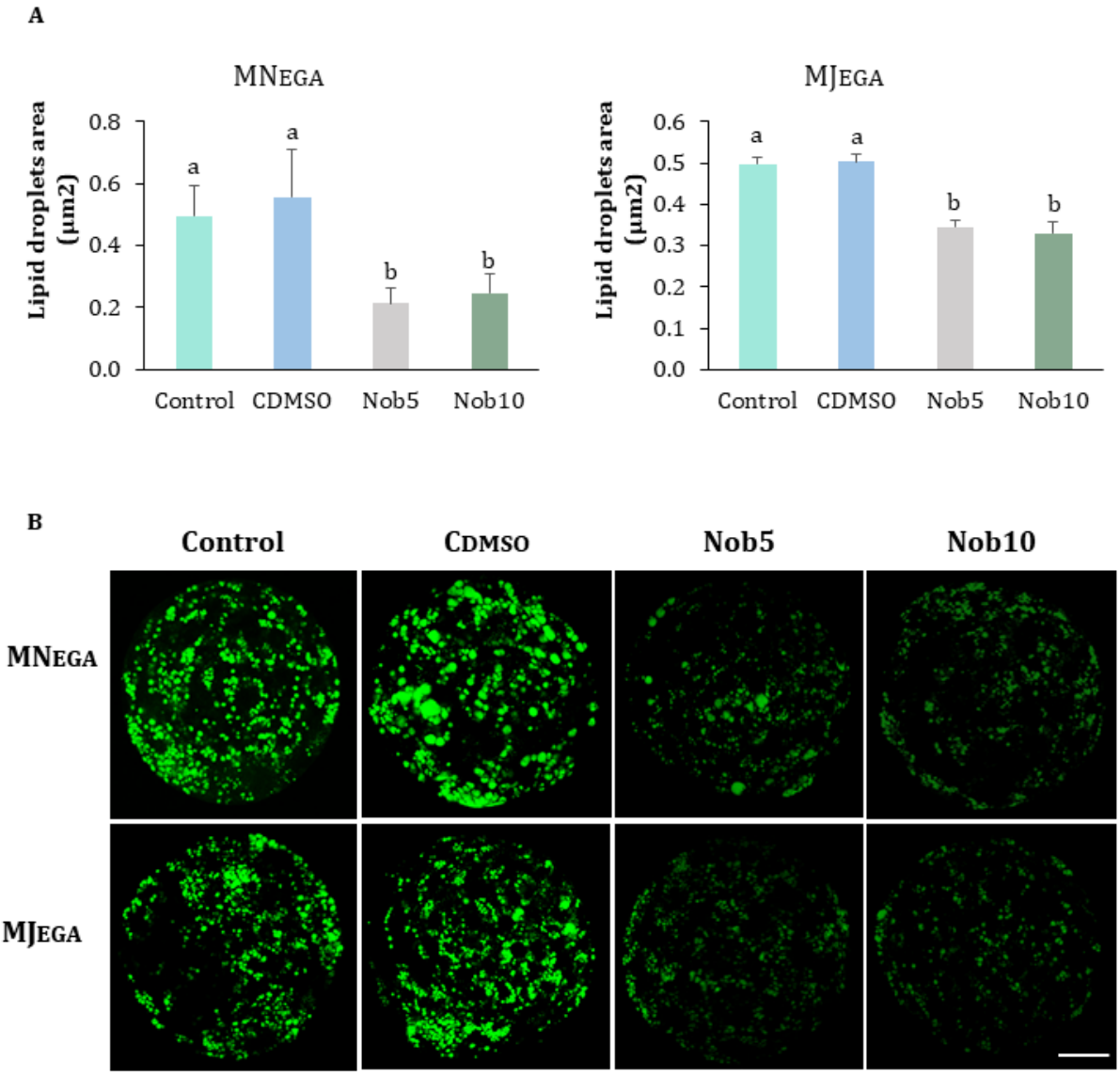

Figure 22. Lipid content in *in vitro* produced bovine blastocyst cultured during 21 - 54 hpi (MNEGA: presumptive zygote to 8-cell stage) or during 54 - 96 hpi (MJEGA: 8- to 16-cell stage) with or without nobiletin. (A) Quantification of the total area of lipid droplets (µm²) in Day 7 blastocysts cultured in SOF+5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (CDMSO) during MNEGA or MJEGA phase. Data are the mean±s.e.m. Significant differences (P<0.001) are indicated with different letters. (B) Representative fluorescence images of lipid droplets in Day 7 blastocysts from all experimental groups (Control, Nob5 Nob10, CDMSO) in both phases (MNEGA or MJEGA). Images were captured on 63× objective. Scale bar 50 µm.

The total number cells was greater (P<0.001) in blastocysts from MNEGA phase with 5 µM of nobiletin (137.3 ± 0.6) compared to all other groups (Control: 105.7 ± 0.7; CDMSO: 106.4 ± 0.8; Nob10: 126.7 ± 0.8), while blastocysts from Nob10 group had more cells (P<0.001) compared to control groups, but less (P<0.001) when compared to Nob5. However, during MJEGA phase the total number of cells was higher in blastocysts from Nob5 and Nob10 groups (133.2 ± 0.9 and 134.2 ± 0.7, respectively) compared to control groups (Control: 104.9 ± 0.7 and CDMSO: 104.6 ± 0.6) (P<0.001) (Table 7).

Table 7. Total nuclei number in *in vitro* produced bovine blastocysts cultured during 21 - 54 hpi (MNEGA: presumptive zygote to 8-cell stage) or during 54 - 96 hpi (MJEGA: 8- to 16-cell stage) in SOF+5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) of nobiletin or with 0.03% dimethyl sulfoxide (CDMSO).

	MNEGA		**MJEGA**	
	No. of embryos examined	Total no. of cells/blastocyst	No. of embryos examined	Total no. of cells/blastocyst
Control	27	105.7 ± 0.7[a]	33	104.9 ± 0.7[a]
CDMSO	26	106.4 ± 0.8[a]	30	104.6 ± 0.6[a]
Nob5	25	137.3 ± 0.6[b]	36	133.2 ± 0.9[b]
Nob10	29	126.7 ± 0.8[c]	34	134.2 ± 0.7[b]

MNEGA: embryos cultured from zygotes to 8-cell stage (21–54 hpi) and MJEGA: embryos cultured from 8-cell to 16-cell stage (54–96 hpi) in SOF+5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) of nobiletin or with 0.03% dimethyl sulfoxide (CDMSO). Data are the mean ± s.e.m. Within columns, different superscript letters indicate significant difference (P<0.05) between treatments.

- **Gene expression in ≥ 8-cell embryos and blastocysts produced with nobiletin during MNEGA**

The mRNA abundance of *CDK2*, *H3-3B*, *H3-3A*, and *GPX1* was significantly increased in 8-cell stage embryos from Nob5 and Nob10 groups compared to both controls (P<0.05) (Figure 23A). The expression of *PPARα* and *GPX1* was significantly higher in blastocysts from Nob5 and Nob10 groups when compared with both controls (P<0.05) (Figure 23B). No differences were observed for the *PPARGC1A*, *PPARα*, *RPS6KB1*, and *NFE2L2* transcripts in 8-cell stage embryos and *PPARGC1A*, *RPS6KB1*, *CDK2*, *H3-3B*, *H3F3A*, and *NFE2L2* in blastocysts.

- **Gene expression in ≥ 16-cell embryos and blastocysts produced with nobiletin during MJEGA**

The expression level of *CDK2, H3-3B* and *NFE2L2* transcripts was significantly increased in 16-cell stage embryos from Nob10 group compared to Nob5 and both control groups. While

the expression of *GPX1* gene was higher in Nob5 and Nob10 compared to control groups (P<0.05) (Figure 24A). In blastocysts the expression of *PPARα* was significantly higher in Nob10 group compared to all other groups (P< 0.05), while *CDK2* and *GPX1* were upregulated in both nobiletin groups compared with controls (P<0.05) (Figure 24B). No significant differences were observed for *PPARGC1A, PPARα, RPS6KB1* and *H3-3A* in 16-cell stage embryos, and for *PPARGC1A, RPS6KB1, H3-3B, H3-3A* and *NFE2L2* in blastocysts

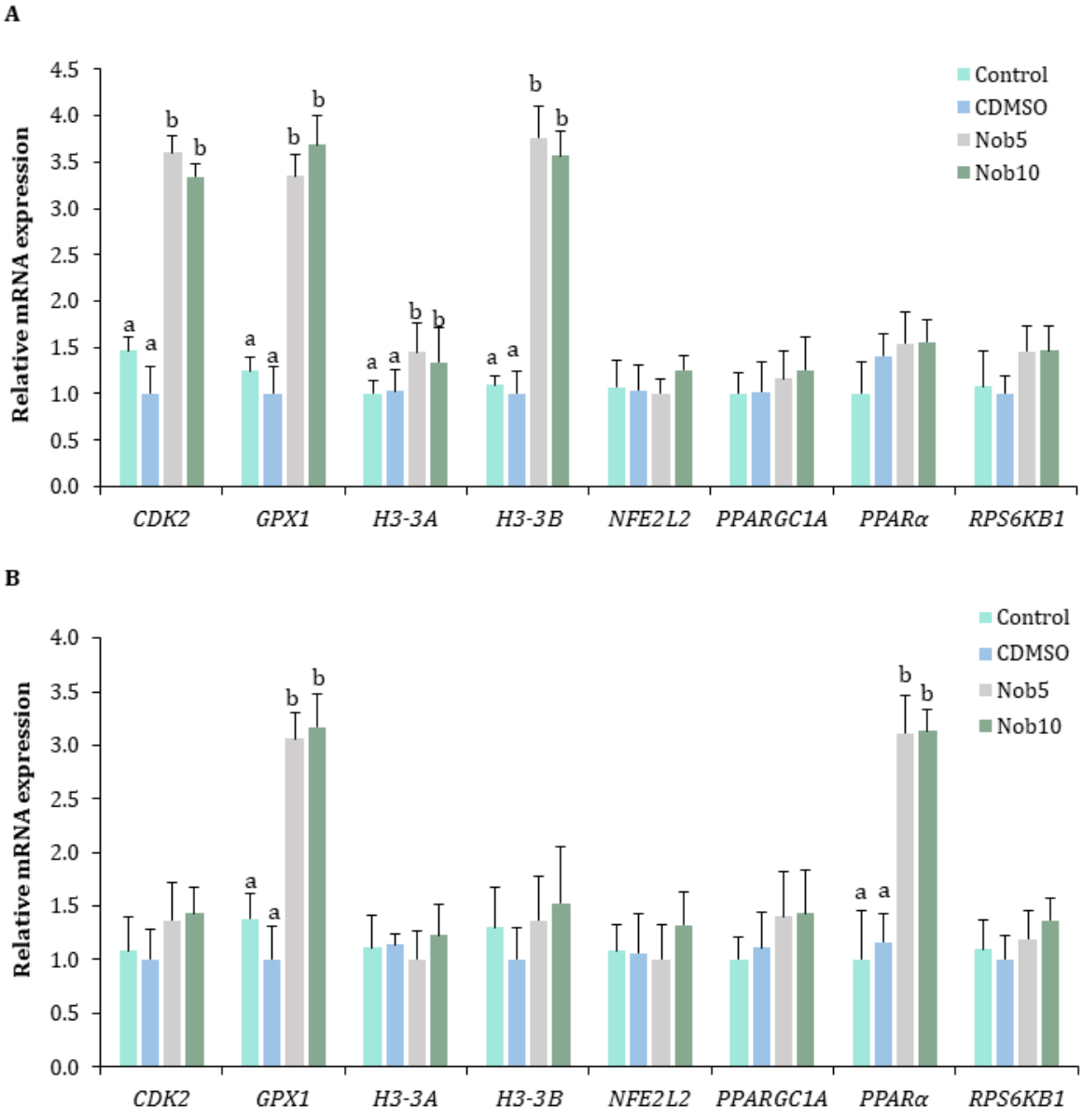

Figure 23. Relative mRNA transcript abundance of embryo development-related genes in *in vitro* produced embryos cultured during 21 - 54 hpi (MNEGA: presumptive zygote to 8-cell stage) with or without nobiletin. (A) Relative abundance in 8-cell stage embryos cultured in SOF+5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (CDMSO) during MNEGA phase. (B) Relative abundance in blastocysts from Control, Nob5, Nob10, and CDMSO experimental groups from MNEGA phase. The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as

housekeeping genes. Data are the mean ± s.e.m. Different letters indicate significant difference (P<0.05) between treatments.

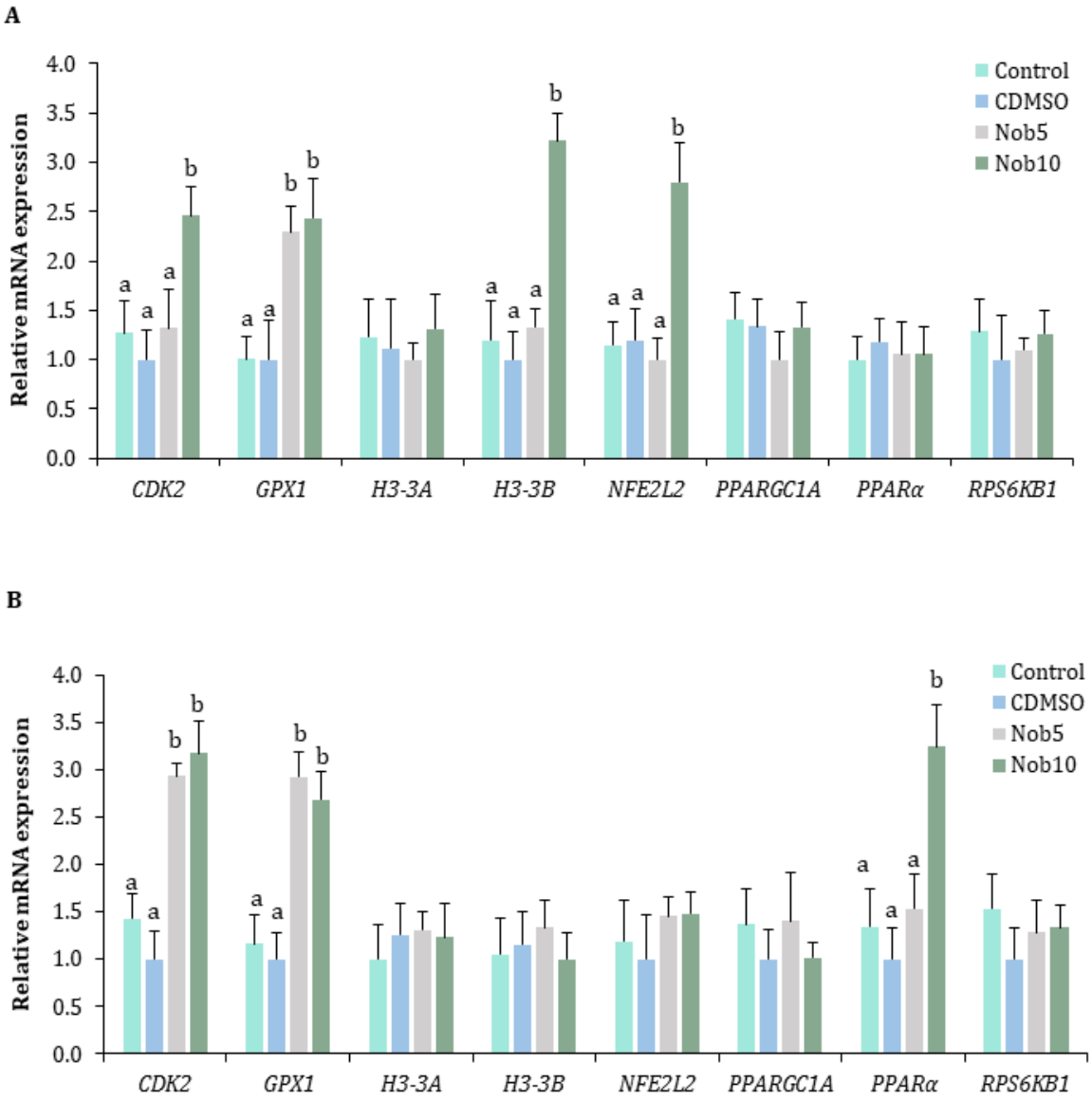

Figure 24. Relative mRNA transcript abundance of embryo development-related genes in *in vitro* produced embryos cultured during 54 - 96 hpi (MJEGA: 8-to 16-cell stage) with or without nobiletin. (A) Relative abundance in16-cell stage embryos cultured in SOF+5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (CDMSO) during MJEGA phase. (B) Relative abundance in blastocysts from Control, Nob5, Nob10, and CDMSO experimental groups from MJEGA phase. The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as housekeeping genes. Data are the mean ± s.e.m. Different letters indicate significant difference (P<0.05) between treatments.

4.2.3. Nobiletin during MNEGA or MJEGA increases AKT phosphorylation in blastocysts produced *in vitro*

Immunofluorescence analysis revealed immunoreactive proteins for p-AKT in bovine blastocysts. In Day 7 blastocysts, AKT increased its phosphorylation levels when nobiletin was present in the culture medium (Nob5 and Nob10 groups) during MNEGA or MJEGA. While p-AKT levels were weaker in blastocysts produced from control groups during MJEGA phase (Figure 25).

Similarly, the western blot analysis showed that both p-AKT-Thr308 and p-AKT-Ser473 phosphorylation levels were significantly higher in blastocysts produced with nobiletin supplementation (Nob5 and Nob10) during MNEGA phase when compared with control groups ($P<0.05$) (Figures 26 A, B, C). A similar pattern was observed in response to nobiletin treatment during MJEGA, as p-AKT-Thr308 and p-AKT-Ser473 phosphorylation levels were significantly higher in blastocysts produced with Nob5 and Nob10 compared with control groups ($P<0.05$) (Figures 26 D, E, F).

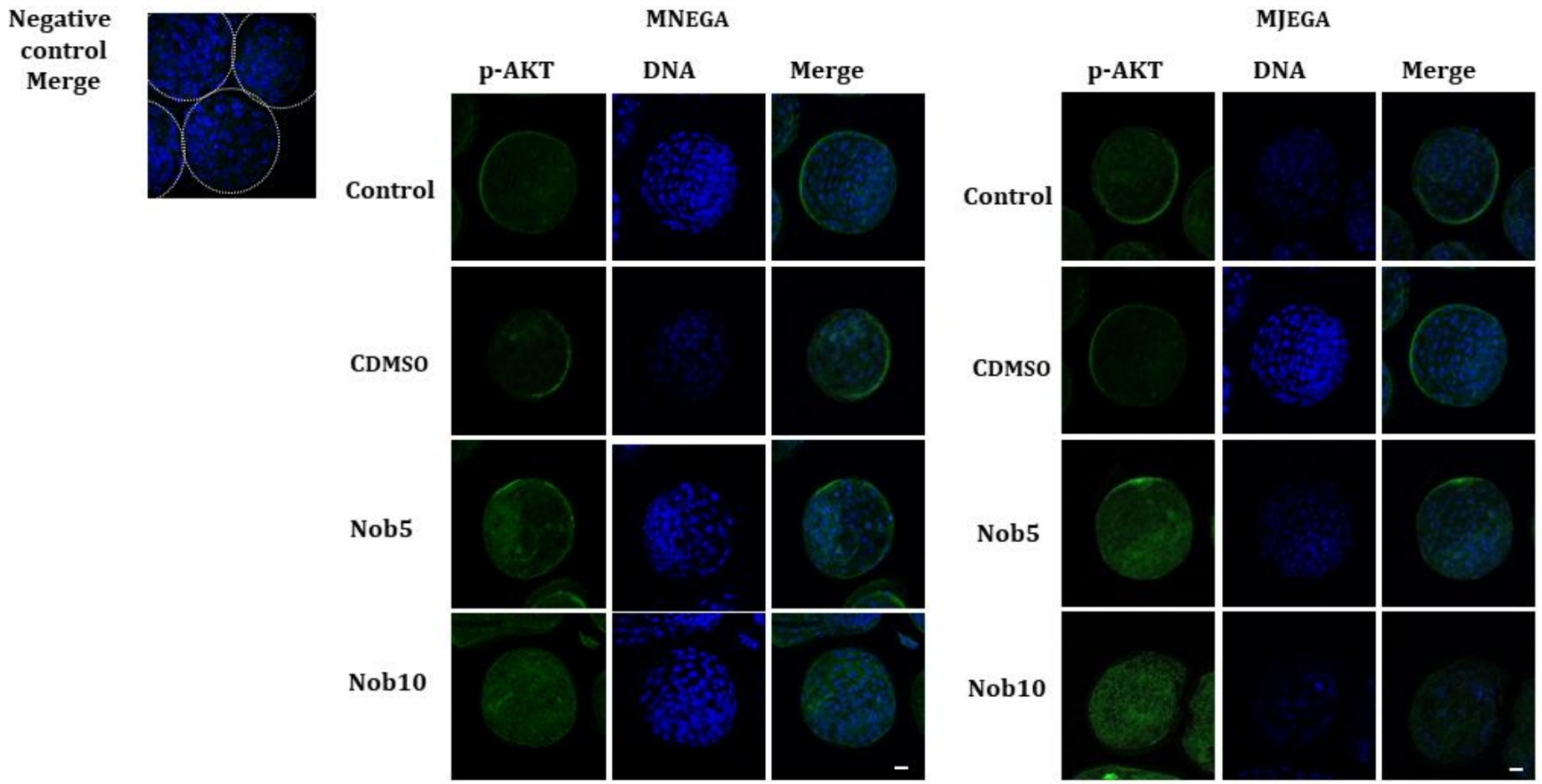

Figure 25. Representative images of immunofluorescence detection of phosphorylated AKT (p-AKT) in *in vitro* produced bovine blastocysts cultured during 21 - 54 hpi (MNEGA: presumptive zygote to 8-cell stage) or during 54 - 96 hpi (MJEGA: 8- to 16-cell stage) in SOF+5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (CDMSO). Positive staining for p-AKT proteins shown in green. Cell nuclei were counterstained with Hoechst stain (blue). Images were captured on 63× objective. Scale bar 20 µm.

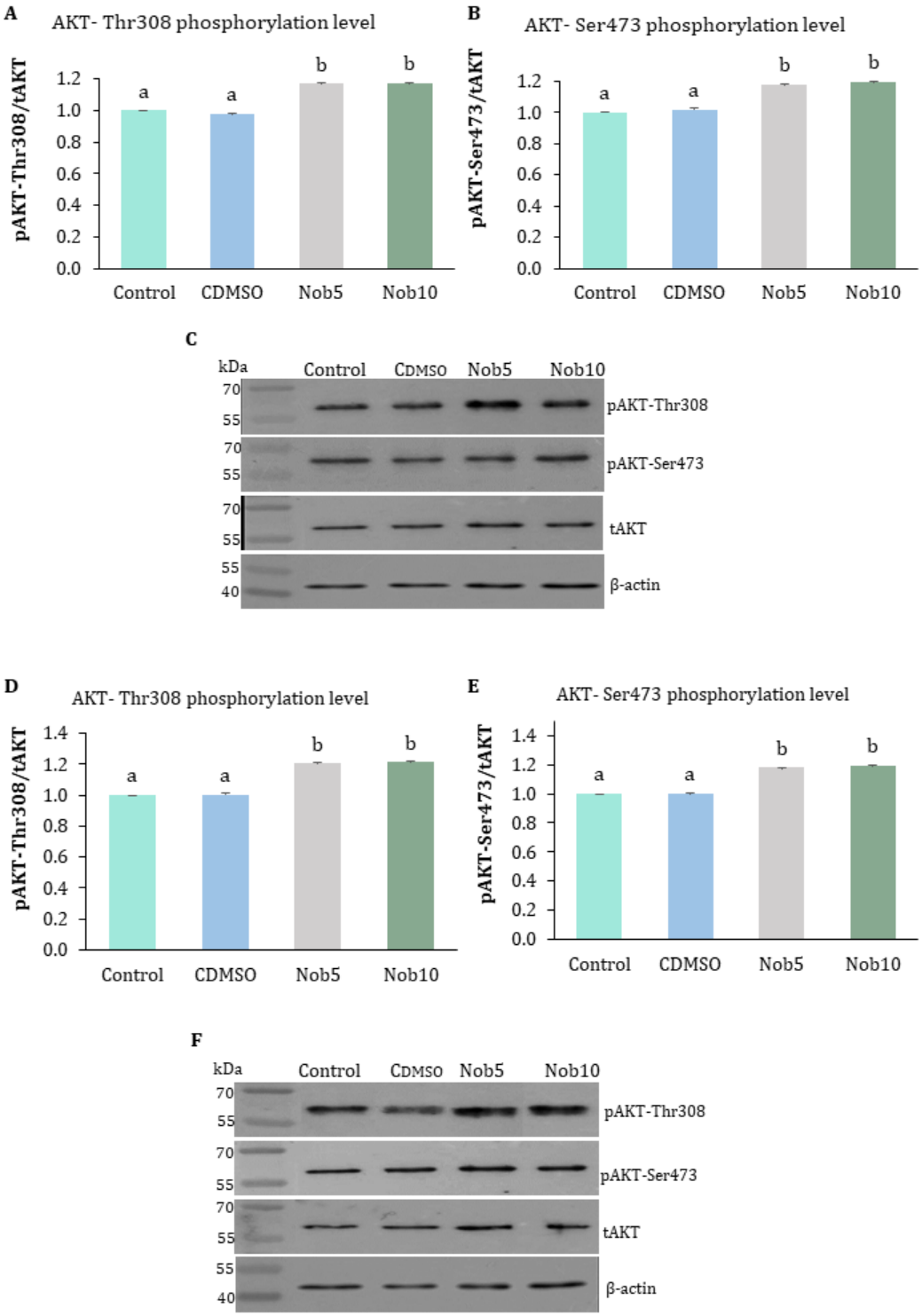

Figure 26. Representative Western blot images of phosphorylated (p)AKT-Thr308, (p)AKT-Ser473, total AKT (tAKT) and β-actin protein levels in *in vitro* produced bovine Day 7 blastocysts cultured during 21 - 54 hpi (MNEGA: presumptive zygote to 8-cell stage) or during 54 - 96 hpi (MJEGA: 8- to 16-cell stage) in SOF+5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (CDMSO). (A) pAKT-Thr308/tAKT, (B) pAKT-Ser473/tAKT (C) Western blot images showing the expression of pAKT-Thr308, pAKT-Ser473, (t)AKT and β-actin in D7 blastocysts from MNEGA phase. (D) pAKT-Thr308/tAKT, (E) pAKT-Ser473/tAKT and (F) Western blot images showing the expression of pAKT-Thr308, pAKT-Ser473, (t)AKT and β-actin in Day 7 blastocysts from from MJEGA phase. Data were normalized relative to the abundance of β-actin and p-AKT phosphorylation levels. Data are the mean ± s.e.m. Different letters indicate significant difference ($P<0.05$) between treatments.

Experiment 3

Can nobiletin reverse the detrimental effect of AKT inhibitors during early embryonic development?

4.3.1. Effect of AKT inhibitors III and IV on early embryo development and their AKT phosphorylation

In MNEGA, no differences were observed in total cleavage rate at 54 hpi, which ranged from 85.3 ± 0.3% to 86.7 ± 0.9%. At 54 hpi, in the treatments with AKT inhibitors, a significantly lower proportion of embryos reached the 8-cell stage that control groups (Inh III: 36.2 ± 1.1% and Inh IV: 29.8 ± 1.2% vs Control: 60.9 ± 1.1% and CDMSO: 60.1 ± 1.3%; (P<0.001)). Similarly, the blastocyst yield at Day 7 was significantly lower (P<0.001) for Inh III (18.3 ± 0.9%) and Inh IV (14.6 ± 1.5%) compared to both controls (Control: 33.7 ± 1.6% and CDMSO: 33.9 ± 2.1%) (Table 8). These differences were maintained also at Day 8 (Table 8).

Table 8. Cleavage rate and kinetics of development at 54 hpi and cumulative blastocyst rates on Days 7 and 8 after *in vitro* culture with or without AKT inhibitors III or IV during MNEGA

	IVC N	Total cleaved 54 hpi N (% ± s.e.m.)	< 8 cells N (% ± s.e.m.)	≥ 8 cells N (% ± s.e.m.)	IVC 54 hpi N	Blastocysts	
						Day 7 N (% ± s.e.m.)	Day 8 N (% ± s.e.m.)
Control	212	184 (86.7 ± 0.9)	55 (25.8 ± 1.7)[a]	129 (60.9 ± 1.1)[a]	99	33 (33.7 ± 1.6)[a]	39 (39.9 ± 2.5)[a]
CDMSO	216	187 (86.6 ± 0.9)	57 (26.5 ± 2.2)[a]	130 (60.1 ± 1.3)[a]	95	32 (33.9 ± 2.1)[a]	37 (38.9 ± 1.0)[a]
Inh III	293	250 (85.3 ± 0.3)	144 (49.1 ± 0.8)[b]	106 (36.2 ± 1.1)[b]	76	14 (18.3 ± 0.9)[b]	15 (19.7 ± 1.1)[b]
Inh IV	317	284 (86.6 ± 0.1)	186 (56.7 ± 1.1)[c]	98 (29.8 ± 1.2)[c]	68	10 (14.6 ± 1.5)[b]	12 (17.7 ± 0.4)[b]

MNEGA: embryos cultured from zygotes to 8-cell stage (21–54 hpi) in SOF+5% FCS (Control), supplemented or not with 15 μM of AKT Inhibitor III (Inh III) or with 10 μM of AKT Inhibitor IV (Inh IV), or with 0.03% dimethyl sulfoxide (CDMSO). Data are the mean ± s.e.m. Within columns, different superscript letters indicate significant difference (P<0.001) between treatments.

During MJEGA, the cleavage rate at 54 hpi was similar for all groups (≈88.5%) as well as the proportion of embryos that reached the 8-cell stage (range: 65.9 ± 0.7% - 67.8 ± 1.1%). At 96 hpi, the proportion of embryos reaching the 16-cell stage was significantly lower (P<0.001) for Inh III (43.3 ± 0.8%) and Inh IV (37.0 ± 0.9%) compared to control groups (Control: 71.9 ± 0.8%; CDMSO: 71.0 ± 0.6%). On Day 7 and 8, blastocyst yield was significantly lower (P<0.001) for Inh III (23.0 ± 1.6% - 24.6 ± 0.8%) and Inh IV (18.6 ± 1.5% and 20.7 ± 1.9%, respectively) compared to Control (35.0 ± 1.0% - 41.6 ± 0.9%) and CDMSO (35.0 ± 1.0% - 41.2 ± 1.5%) (Table 9).

Table 9. Cleavage rate and kinetics of development at 54 and 96 hpi and cumulative blastocyst rates on Days 7 and 8 after *in vitro* culture with or without AKT inhibitors III or IV during MJEGA.

	IVC N	Total cleaved 54 hpi N (% ± s.e.m.)	< 8 cells N (% ± s.e.m.)	≥ 8 cells N (% ± s.e.m.)	IVC 54 hpi N	< 16 cells N (% ± s.e.m.)	≥ 16 cells N (% ± s.e.m.)	IVC 96 hpi N	Blastocysts	
									Day 7 N (% ± s.e.m.)	Day 8 N (% ± s.e.m.)
Control	210	186 (88.6 ± 0.1)	47 (22.4 ± 0.9)	139 (66.2 ± 0.8)	139	39 (28.1 ± 0.8)[a]	100 (71.9 ± 0.8)[a]	60	21 (35.0 ± 1.0)[a]	25 (41.6 ± 0.9)[a]
CDMSO	220	195 (88.6 ± 0.5)	50 (22.7 ± 0.2)	145 (65.9 ± 0.7)	145	42 (28.9 ± 0.6)[a]	103 (71.0 ± 0.6)[a]	63	22 (35.0 ± 1.0)[a]	26 (41.2 ± 1.5)[a]
Inh III	277	245 (88.8 ± 1.2)	58 (20.9 ± 0.7)	187 (67.4 ± 1.6)	187	106 (56.7 ± 0.8)[b]	81 (43.3 ± 0.8)[b]	61	14 (23.0 ± 1.6)[b]	15 (24.6 ± 0.8)[b]
Inh IV	273	243 (89.4 ± 0.5)	58 (21.3 ± 1.0)	185 (67.8 ± 1.1)	185	116 (63.0 ± 0.9)[c]	68 (37.0 ± 0.9)[c]	48	9 (18.6 ± 1.5)[b]	10 (20.7 ± 1.9)[b]

MJEGA: embryos cultured from 8-cell to 16-cell stage (54–96 hpi) in SOF+5% FCS (Control), supplemented or not with 15 µM of AKT Inhibitor III (Inh III) or with 10 µM of AKT Inhibitor IV (Inh IV), or with 0.03% dimethyl sulfoxide (CDMSO). Data are the mean ± s.e.m. Within columns, different superscript letters indicate significant difference (P<0.001) between treatments.

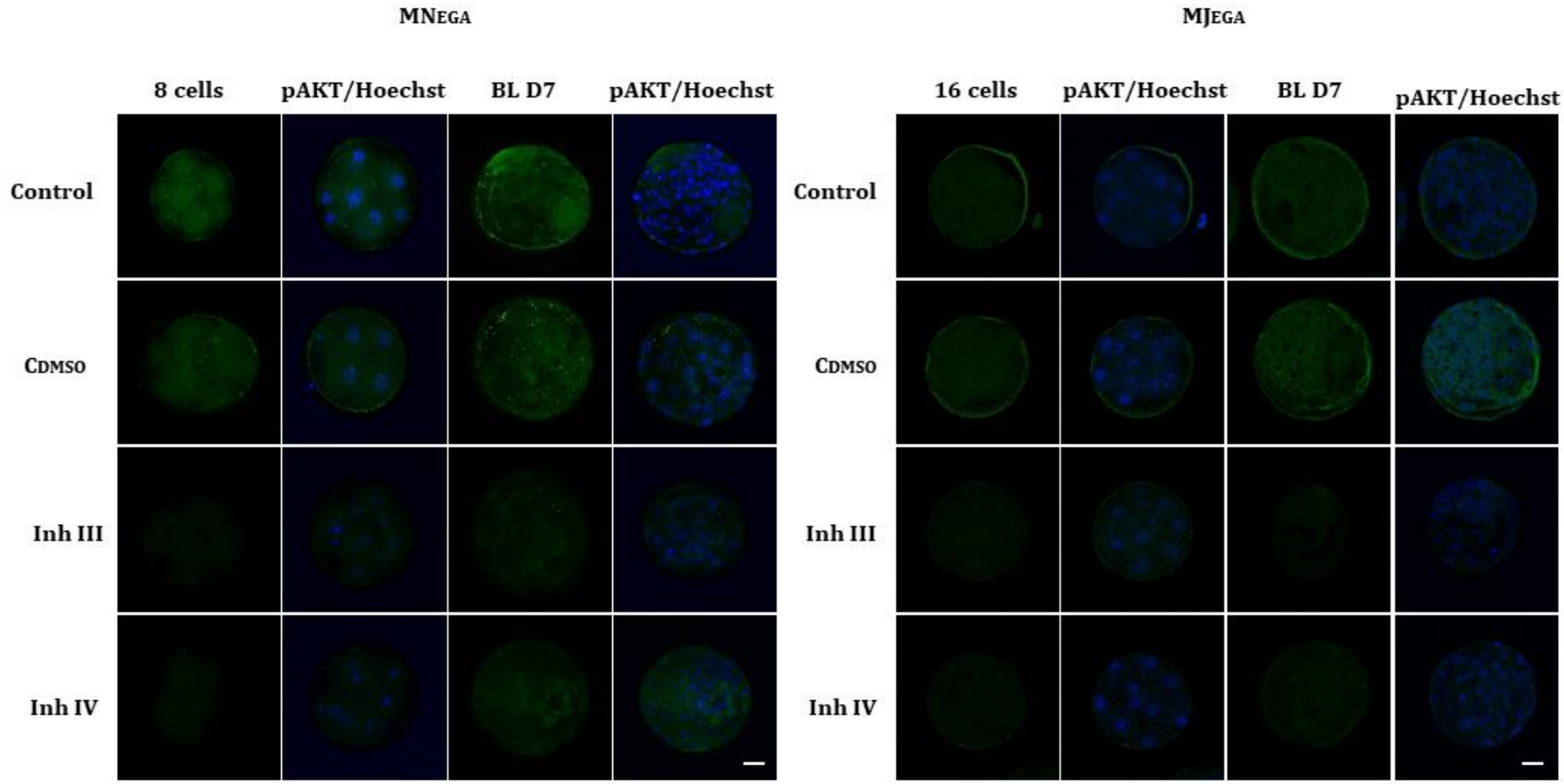

Figure 27. Representative images of immunofluorescence phosphorylation of AKT (Thr308) in *in vitro* produced bovine 8-, 16-cell stage embryos and blastocysts cultured during 21 - 54 hpi (MNEGA: presumptive zygote to 8-cell stage) or during 54 - 96 hpi (MJEGA: 8- to 16-cell stage) in SOF with 5% FCS (Control); supplemented or not with 15 µM of AKT Inhibitor III (Inh III) or with 10 µM of AKT Inhibitor IV (Inh IV), or with 0.03% dimethyl sulfoxide (DMSO vehicle for nobiletin and AKT Inhibitors dilution (CDMSO)). Positive staining for p-AKT proteins shown in green. Cell nuclei were counterstained with Hoechst stain (blue). Images were captured on 63× objective. Scale bar 20 µm.

Immunofluorescence analysis revealed immunoreactive proteins for p-AKT in 8-cell embryos (MNEGA), 16-cell embryos (MJEGA) and bovine Day 7 blastocysts from both EGA phases. During MNEGA, AKT significantly decreased its phosphorylation intensity in 8-cell stage embryos and blastocysts in presence of AKT inhibitor. Even so, the 8-cell stage embryos had the weakest signal not only in 8-cell embryos but also in the blastocysts produced with AKT inhibitors (Figure 27). Similar phosphorylation intensity was observed during MJEGA, with significantly decreased phosphorylation signal in 16-cell embryos and blastocysts produced with AKT inhibitors. However, for Inh III the phosphorylation signal in blastocysts was weaker compared to control groups and Inh IV (Figure 27).

4.3.2. Recovery of the adverse effects of AKT inhibition on early embryo development with nobiletin supplementation during MNEGA or MJEGA

For all experimental groups, only embryos reached the 8-cell stage at 54 hpi were selected for the study. As shown in Figure 28A and Table 10 for MNEGA phase, cleavage rate was not different between groups (range: 86.1 ± 0.3% to 88.0 ± 0.4%). At 54 hpi, nobiletin supplementation (66.2 ± 0.4%) significantly increased ($P<0.001$) the proportion of embryos reaching 8-cell stage compared to all other groups (range: 31.1 ± 0.3% to 61.4 ± 0.4%); while, supplementation of Inh III (38.0 ± 0.3%) and Inh IV (31.1 ± 0.3%) significantly decreases ($P<0.001$) the proportion of embryos reaching 8-cell stage compared to all other groups (range: 46.0 ± 0.5% to 66.2 ± 0.4%) (Figure 28B, Table 10). However, combination of nobiletin with AKT inhibitors retrieve their adverse effect in terms of the proportion of 8 cell stage embryos with Nob+Inh III group (59.2 ± 0.5%) being similar to Control (61.4 ± 0.4%) and CDMSO (60.8 ± 0.5%) groups, while Nob+Inh IV group (46.0 ± 0.5%) was intermediate (Figure 28B, Table 10).

Similarly, blastocyst yield at Day 7 and 8 (Figure 28C, Table 10) was significantly higher ($P<0.001$) for Nob group (42.5 ± 0.8% and 46.2 ± 1.2%, respectively) compared to the rest of the groups (range: 14.9 ± 0.8% to 34.2 ± 1.2% and 19.0 ± 0.8% to 38.6 ± 1.7%, respectively); while for Inh III (19.6 ± 0.9% and 21.6 ± 0.8%, respectively) and Inh IV (14.9 ± 0.8% and 19.0 ± 0.8%, respectively) groups was significantly lower ($P<0.001$) to both controls and Nob+Inh III groups (Figure 28C, Table 10). Nevertheless, nobiletin was able to retrieve the adverse developmental effect of AKT Inhibitor III in blastocyst yield on Day 7 and 8 (34.2 ± 1.2% and 38.6 ± 1.2%, respectively) being similar to Control (33.9 ± 0.9% and 38.6 ± 1.7%, respectively) and CDMSO (33.5 ± 0.9% and 37.6 ± 1.7%, respectively) groups, while for Inh IV the effect was limited (Figure 28C, Table 10).

Table 10. Cleavage rate and kinetics of development at 54 hpi and cumulative blastocyst rates on Days 7 and 8 after *in vitro* culture with or without nobiletin and/or AKT inhibitors supplemented during MNEGA.

	IVC N	Total cleaved 54 hpi N (% ± s.e.m.)	< 8 cells N (% ± s.e.m.)	≥ 8 cells N (% ± s.e.m.)	IVC 54 hpi N	Blastocysts	
						Day 7 N (% ± s.e.m.)	Day 8 N (% ± s.e.m.)
Control	560	483 (86.4 ± 0.7)	139 (24.8 ± 0.8)[a]	344 (61.4 ± 0.4)[a]	224	76 (33.9 ± 0.9)[a]	86 (38.6 ± 1.7)[a]
CDMSO	597	514 (86.1 ± 0.6)	151 (25.3 ± 0.7)[a]	363 (60.8 ± 0.5)[a]	243	81 (33.5 ± 0.9)[a]	92 (37.6 ± 1.7)[a]
Inh III	818	705 (86.1 ± 0.3)	395 (48.0 ± 0.6)[b]	310 (38.0 ± 0.3)[b]	190	37 (19.6 ± 0.9)[b]	41 (21.6 ± 0.8)[bc]
Inh IV	948	828 (87.2 ± 0.3)	534 (56.1 ± 0.5)[c]	294 (31.1 ± 0.3)[c]	174	27 (14.9 ± 0.8)[c]	33 (19.0 ± 0.8)[b]
Nob	660	581 (88.0 ± 0.4)	144 (21.8 ± 0.4)[d]	437 (66.2 ± 0.4)[d]	317	133 (42.5 ± 0.8)[d]	145 (46.2 ± 1.2)[d]
Nob+Inh III	549	476 (86.6 ± 0.6)	151 (27.5 ± 0.7)[a]	325 (59.2 ± 0.5)[a]	205	70 (34.2 ± 1.2)[a]	79 (38.6 ± 1.2)[a]
Nob+Inh IV	651	574 (87.8 ± 0.3)	273 (41.9 ± 0.4)[e]	30 1 (46.0 ±0.5)[e]	181	37 (21.1 ± 1.6)[b]	44 (24.6 ± 0.9)[c]

MNEGA: embryos cultured from zygotes to 8-cell stage (21–54 hpi) in SOF+5% FCS (Control), supplemented or not with 15 μM of AKT Inhibitor III (Inh III) or with 10 μM of AKT Inhibitor IV (Inh IV), or with 10 μM nobiletin (Nob), or with 10 μM nobiletin + 15 μM of AKT Inhibitor III (Nob+Inh III), or with 10 μM nobiletin + 10 μM of AKT Inhibitor IV (Nob+Inh IV), or with 0.03% dimethyl sulfoxide (DMSO vehicle for nobiletin and AKT Inhibitors dilution (CDMSO)). Data are the mean ± s.e.m. Within columns, different superscript letters indicate significant difference (P<0.001) between treatments.

As shown in Figure 28D and Table 11, during MJEGA cleavage rate at 54 hpi was similar for all groups (range: 86.2 ± 0.6% to 87.0 ± 0.5%) and the proportion of embryos that reached the 8-cell stage was around 65.0% for all groups as expected. At 96 hpi, the proportion of embryos reaching 16-cell was significantly higher (P<0.001) for Control (71.6 ± 0.6%), CDMSO (69.8 ± 0.9%) and Nob (68.6 ± 0.7%) compared to Inh III (42.7 ± 0.3%), Inh IV (35.2 ± 0.4%), Nob+Inh III (58.6 ± 0.5%), and Nob+Inh IV (54.7 ± 0.6%) groups; while Nob was significantly lower (P<0.001) to Control but similar to CDMSO (Figure 28E, Table 11). As expected, supplementation of AKT Inhibitors decrease (P<0.001) the proportion of embryos reaching 16-cell stage compared to all other groups. However, combination of nobiletin with AKT inhibitors increase (P<0.001) the proportion of 16-cell stage embryos compared to AKT inhibitors alone but still being lower (P<0.001) to the remaining groups (Figure 28E, Table 11).

Blastocyst yield at Day 7 and 8 (Figure 28F, Table 11) was significantly higher (P<0.001) for Nob group (57.6 ± 1.7% and 62.4 ± 1.6%, respectively) compared to the rest of the groups (range: 17.2 ± 0.8% to 38.8 ± 1.5% and 22.7 ± 0.9% to 44.0 ± 1.9%, respectively); while for Inh III (22.9 ± 1.3% and 28.9 ± 0.6%, respectively) and Inh IV (17.2 ± 0.8% and 22.7 ± 0.9%, respectively) groups was significantly lower (P<0.001) to both controls and Nob+Inh III groups (Figure 28E, Table 11). Nevertheless, as in MNEGA phase, nobiletin was able to retrieve the adverse developmental effect of AKT Inhibitor III in blastocyst yield on Day 7 and 8 (38.8 ± 1.5% and 42.1 ± 1.7%, respectively) being similar to Control (36.7 ± 0.8% and 41.7 ± 0.8%, respectively) and CDMSO (38.6 ± 1.1% and 44.0 ± 1.9%, respectively) groups, while for Inh IV the effect was limited (Figure 28F, Table 11).

Table 11. Cleavage rate and kinetics of development at 54 and 96 hpi and cumulative blastocyst rates on Days 7 and 8 after *in vitro* culture with or without nobiletin and/or AKT inhibitors supplemented during MJEGA.

	IVC N	Total cleaved 54 hpi N (% ± s.e.m.)	< 8 cells N (% ± s.e.m.)	≥ 8 cells N (% ± s.e.m.)	IVC 54 hpi N	< 16 cells N (% ± s.e.m.)	≥ 16 cells N (% ± s.e.m)	IVC 96 hpi N	Blastocysts	
									Day 7 N (% ± s.e.m.)	Day 8 N (% ± s.e.m.)
Control	594	531 (87.0 ± 0.5)	140 (23.5 ± 0.6)^{a}	391 (65.9 ± 0.6)^{a}	391	110 (28.3 ± 0.7)^{a}	281 (71.6 ± 0.6)^{a}	161	62 (36.7 ± 0.8)^{a}	71 (41.7 ± 0.8)^{a}
CDMSO	651	581 (86.8 ± 0.7)	164 (25.2 ± 0.4)^{ab}	417 (64.0 ± 0.4)^{ab}	417	126 (30.2 ± 0.9)^{ad}	291 (69.8 ± 0.9)^{ad}	171	64 (38.6 ± 1.1)^{a}	75 (44.0 ± 1.9)^{a}
Inh III	1080	965 (86.4 ± 0.5)	278 (25.8 ± 0.5)^{ab}	687 (63.5 ± 0.5)^{ab}	687	394 (57.4 ± 0.3)^{b}	293 (42.7 ± 0.3)^{b}	173	44 (22.9 ± 1.3)^{b}	48 (28.9 ± 0.6)^{b}
Inh IV	1109	997 (86.3 ± 0.4)	295 (26.6 ± 0.5)^{b}	702 (63.3 ± 0.5)^{b}	702	166 (65.0 ± 0.3)^{c}	387 (35.2 ± 0.4)^{c}	134	27 (17.2 ± 0.8)^{c}	30 (22.7 ± 0.9)^{c}
Nob	639	569 (86.2 ± 0.6)	129 (20.2 ± 0.3)^{c}	440 (68.9 ± 0.3)^{c}	440	138 (31.4 ± 0.7)^{d}	302 (68.6 ± 0.7)^{d}	182	109 (57.6 ± 1.7)^{d}	120 (62.4 ± 1.6)^{d}
Nob+Inh III	821	734 (86.2 ± 0.6)	205 (25.3 ± 0.3)^{ab}	529 (64.0 ± 0.4)^{ab}	529	215 (41.4 ± 0.5)^{e}	304 (58.6 ± 0.5)^{e}	184	72 (38.8 ± 1.5)^{a}	81 (42.1 ± 1.7)^{a}
Nob+Inh IV	849	753 (86.2 ± 0.6)	203 (23.9 ± 0.3)^{a}	550 (64.8 ± 0.5)^{ab}	550	250 (45.5 ± 0.5)^{f}	300 (54.7 ± 0.6)^{f}	205	48 (25.7 ± 1.1)^{b}	56 (33.5 ± 1.3)^{b}

MJEGA: embryos cultured from 8-cell to 16-cell stage (54-96 hpi) in SOF+5% FCS (Control), supplemented or not with 15 µM of AKT Inhibitor III (Inh III) or with 10 µM of AKT Inhibitor IV (Inh IV), or with 10 µM nobiletin (Nob), or with 10 µM nobiletin + 15 µM of AKT Inhibitor III (Nob+Inh III), or with 10 µM nobiletin + 10 µM of AKT Inhibitor IV (Nob+Inh IV), or with 0.03% dimethyl sulfoxide (DMSO vehicle for nobiletin and AKT Inhibitors dilution (CDMSO)). Data are the mean ± s.e.m. Within columns, different superscript letters indicate significant difference (P<0.001) between treatments.

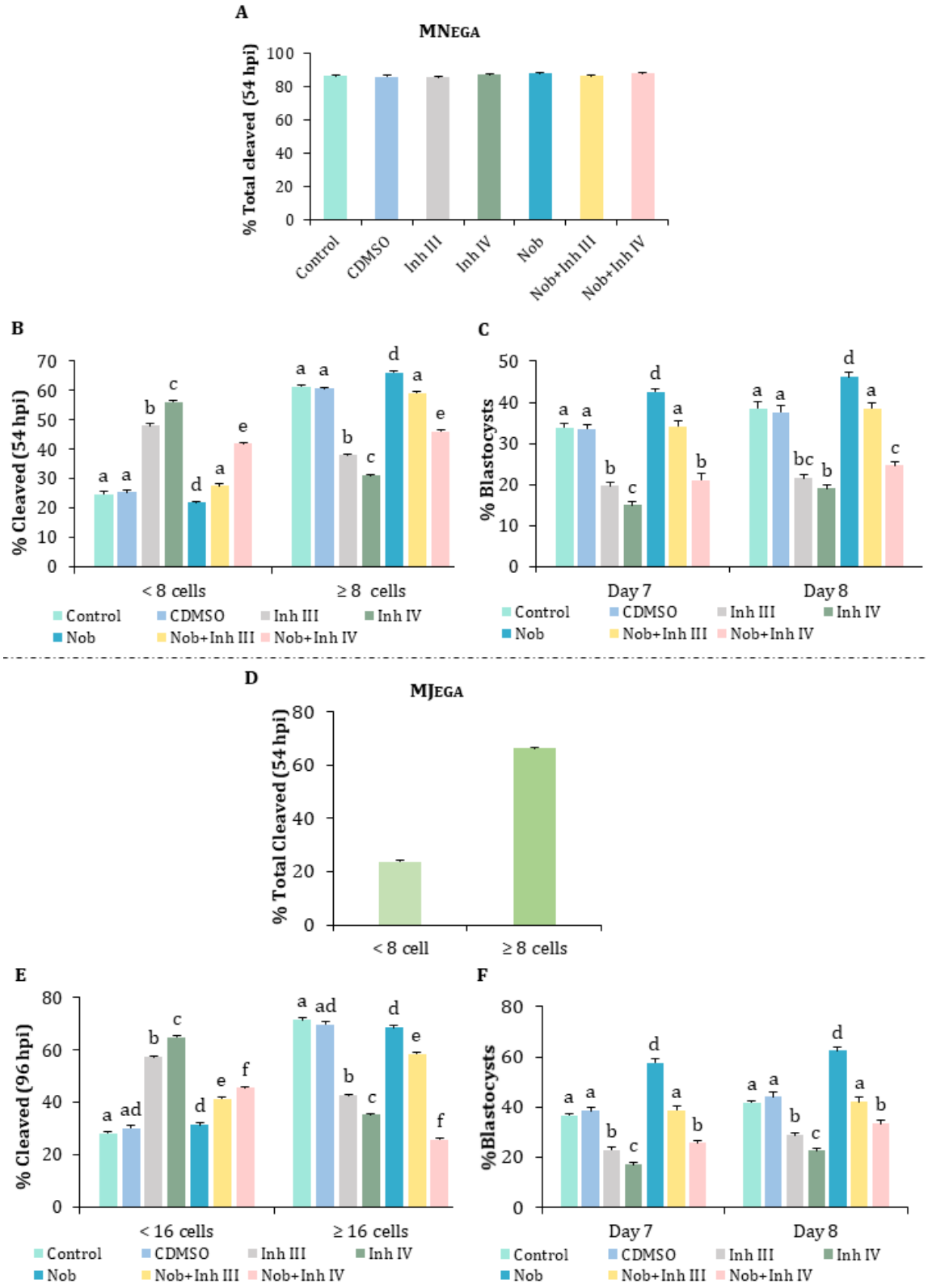

Figure 28. Developmental rates of *in vitro* produced bovine embryos cultured during 21 - 54 hpi (MNEGA: A, B, C) or during 54 - 96 hpi (MJEGA: D, E, F) in SOF with 5% FCS (Control); supplemented or not with 15 µM of AKT Inhibitor III (Inh III) or with 10 µM of AKT Inhibitor IV (Inh IV), or with 10 µM nobiletin (Nob), or with 10 µM nobiletin + 15 µM of AKT Inhibitor III (Nob+Inh III), or with 10 µM nobiletin + 10 µM of AKT Inhibitor IV (Nob+Inh IV), or with 0.03% dimethyl sulfoxide (DMSO vehicle for nobiletin and AKT Inhibitors dilution (CDMSO)). (A, D) Total cleavage rate at 54 hpi; (B, E) embryos ≥ or < 8-cell stage at 54 hpi and ≥ or < 16-cell stage at 96 hpi; (C, F) blastocyst rate on Days 7-8 pi (*in vitro* fertilization = Day 0). Results are expressed as mean ± s.e.m. Significant differences (P<0.001) are indicated with different letters.

4.3.3. Nobiletin supplementation retrieves the harmful effects of AKT inhibition on early embryo quality during EGA.

As showed in Figure 29A, the mRNA abundance of *GPX1*, *NFE2L2* and *POU5F1* transcripts significantly increased in 8-cell stage embryos (MNEGA) from Nob group, while it was decreased (P<0.05) from AKT inhibitors compared to all other groups. Although, nobiletin supplementation was able to partially recover the adverse effect of AKT inhibitors for these transcripts, their expression was still lower (P<0.05) than controls groups. No differences were observed for *CDX2*, *CHD1*, *DNMT3B* and *KLF4* transcripts (Figure 29A).

As showed in Figure 29B, the mRNA abundance of *CDX2, GPX1*, *NFE2L2*, *POU5F1*, and *FOSL1* transcripts significantly increased in 16-cell stage (MJEGA) from Nob group, while it was decreased (P<0.05) from AKT inhibitors compared to all other groups. Although, nobiletin supplementation was able to partially recover the adverse effect of AKT inhibitors for these transcripts, their expression was still lower (P<0.05) than controls groups. No differences were observed for the *CHD1*, *DNMT3B* and *KLF4* transcripts (Figure 29B).

Nobiletin supplementation during MNEGA or MJEGA significantly increased (P<0.001) the blastocysts total cell number (MNEGA: 161.7 ± 0.3 and MJEGA: 164.7 ± 0.6), whereas AKT Inh III (MNEGA: 95.4 ± 0.6 and MJEGA: 92.8 ± 0.8) and Inh IV (MNEGA: 93.1 ± 1.0 and MJEGA: 78.3 ± 1.0) significantly decreased (P<0.001) the blastocysts total cell number when compared to all other groups (Tables 12, 13).

However, nobiletin was able to recover the adverse effect of AKT inhibitors by increasing the blastocysts total cell number from MNEGA group (Nob+Inh III: 133.1 ± 0.9 and Nob+Inh IV: 129.7 ± 0.3) and MJEGA group (Nob+Inh III: 129.2 ± 0.8 and Nob+Inh IV: 127.2 ± 0.6), although still being significantly lower than control groups. Similar trends were observed for TE and ICM cells (Tables 12,13; Figure 30).

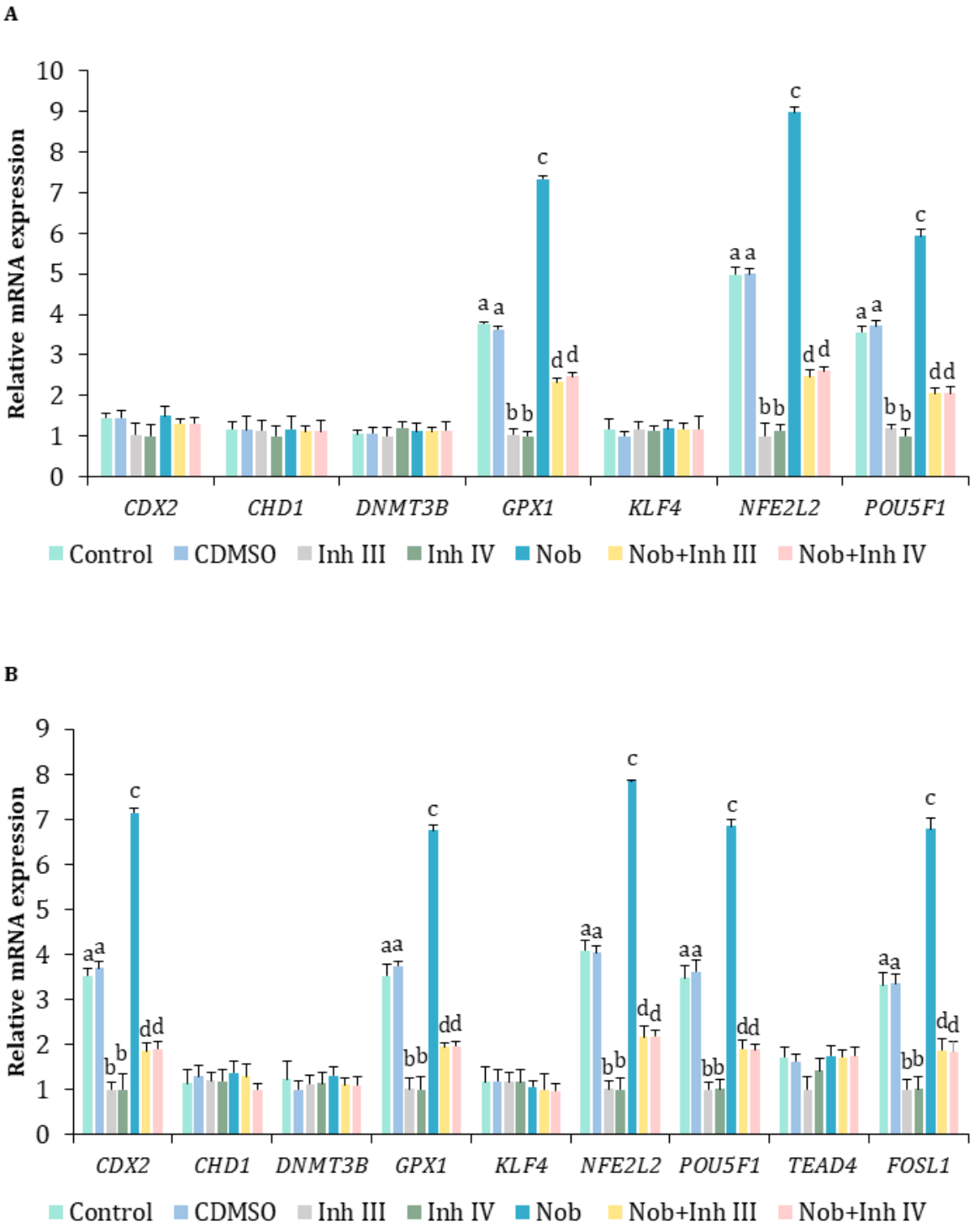

Figure 29. Relative mRNA transcript abundance of embryo development-related genes in *in vitro* produced bovine early embryos. (A) 8-cell stage embryos cultured during 21-54 hpi (MNEGA: presumptive zygotes to 8-cell stage) and (B) 16-cell stage embryos cultured during 54-96 hpi (MJEGA: 8- to 16-cell stage) in SOF with 5% FCS (Control); supplemented or not with 15 µM of AKT Inhibitor III (Inh III) or with 10 µM of AKT Inhibitor IV (Inh IV), or with 10 µM nobiletin (Nob), or with 10 µM nobiletin + 15 µM of AKT Inhibitor III (Nob+Inh III), or with 10 µM nobiletin + 10 µM of AKT Inhibitor IV (Nob+Inh IV), or with 0.03% dimethyl sulfoxide (DMSO vehicle for nobiletin and AKT Inhibitors dilution (CDMSO)). The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as housekeeping genes. Data are the mean±s.e.m. Different letters indicate significant difference (P<0.05) between treatments.

Table 12. Blastocysts cell number after *in vitro* culture with or without nobiletin and/or AKT inhibitors III or IV during MNEGA.

	No. blastocysts processed	No. Total nuclei	No. ICM nuclei	No. TE nuclei	Ratio ICM/TE
Control	31	150.0 ± 0.4[a]	45.4 ± 0.3[a]	104.5 ± 0.4[a]	0.4 ± 0.02
CDMSO	30	148.1 ± 0.4[a]	44.5 ± 0.2[a]	103.6 ± 0.3[a]	0.4 ± 0.01
Inh III	28	95.4 ± 0.6[b]	33.5 ± 0.8[b]	61.8 ± 0.8[b]	0.5 ± 0.11
Inh IV	24	93.1 ± 1.0[b]	31.3 ± 1.0[b]	61.8 ± 1.4[b]	0.5 ± 0.14
Nob	33	161.7 ± 0.3[c]	57.3 ± 0.5[c]	104.4 ± 0.6[a]	0.5 ± 0.04
Nob+Inh III	31	133.1 ± 0.9[d]	42.8 ± 0.3[ad]	90.4 ± 0.7[c]	0.5 ± 0.02
Nob+Inh IV	27	129.7 ± 0.3[e]	42.0 ± 0.2[ad]	87.7 ± 0.2[c]	0.5 ± 0.02

MNEGA: embryos cultured from zygotes to 8-cell stage (21–54 hpi) in SOF+5% FCS (Control), supplemented or not with 15 μM of AKT Inhibitor III (Inh III) or with 10 μM of AKT Inhibitor IV (Inh IV), or with 10 μM nobiletin (Nob), or with 10 μM nobiletin + 15 μM of AKT Inhibitor III (Nob+Inh III), or with 10 μM nobiletin + 10 μM of AKT Inhibitor IV (Nob+Inh IV), or with 0.03% dimethyl sulfoxide (CDMSO). ICM: inner cell mass; TE: trophectoderm. Data are the mean ± s.e.m. Within columns, different superscript letters indicate significant difference (P<0.001) between treatments.

Table 13. Blastocysts cell number after *in vitro* culture with or without nobiletin and/or AKT inhibitors III or IV during MJEGA.

	No. blastocysts processed	No. Total nuclei	No. ICM nuclei	No. TE nuclei	Ratio ICM/TE
Control	34	151.3 ± 0.6[a]	47.5 ± 0.6[a]	103.9 ± 0.9[a]	0.5 ± 0.05
CDMSO	31	150.0 ± 0.6[a]	46.4 ± 0.6[a]	103.6 ± 0.9[a]	0.5 ± 0.05
Inh III	29	92.8 ± 0.8[b]	35.6 ± 0.5[b]	57.4 ± 1.0[b]	0.6 ± 0.09
Inh IV	27	78.3 ± 1.0[c]	33.6 ± 0.5[b]	44.7 ± 0.9[c]	0.7 ± 0.10
Nob	34	164.7 ± 0.6[d]	57.6 ± 0.6[c]	107.1 ± 0.9[d]	0.5 ± 0.05
Nob+Inh III	26	129.2 ± 0.8[e]	52.0 ± 0.6[d]	77.2 ± 1.0[e]	0.7 ± 0.07
Nob+Inh IV	24	127.2 ± 0.6[e]	52.3 ± 0.7[d]	74.9 ± 1.2[e]	0.7 ± 0.10

MJEGA: embryos cultured from 8-cell to 16-cell stage (54-96 hpi) in SOF+5% FCS (Control), supplemented or not with 15 μM of AKT Inhibitor III (Inh III) or with 10 μM of AKT Inhibitor IV (Inh IV), or with 10 μM nobiletin (Nob), or with 10 μM nobiletin + 15 μM of AKT Inhibitor III (Nob+Inh III), or with 10 μM nobiletin + 10 μM of AKT Inhibitor IV (Nob+Inh IV), or with 0.03% dimethyl sulfoxide (CDMSO). ICM: inner cell mass; TE: trophectoderm. Data are the mean ± s.e.m. Within columns, different superscript letters indicate significant difference (P<0.001) between treatments.

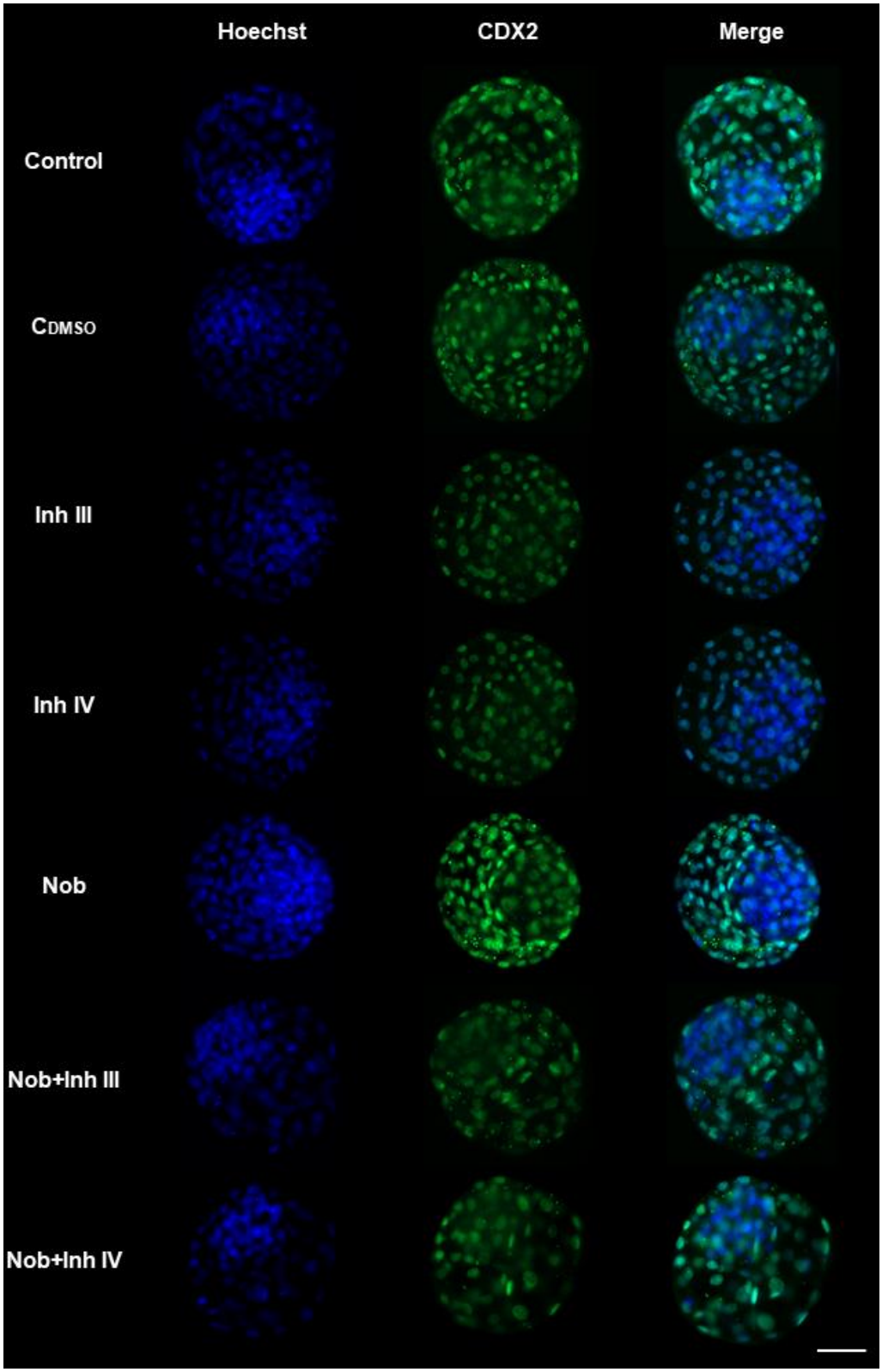

Figure 30. Representative images of bovine blastocyst differential cell count. Embryos cultured from 8-cell to 16-cell stage (MJEGA: 54-96 hpi) in SOF+5% FCS (Control), supplemented or not with 15 µM of AKT Inhibitor III (Inh III) or with 10 µM of AKT Inhibitor IV (Inh IV), or with 10 µM nobiletin (Nob), or with 10 µM nobiletin + 15 µM of AKT Inhibitor III (Nob+Inh III), or with 10 µM nobiletin + 10 µM of AKT Inhibitor IV (Nob+Inh IV), or with 0.03% dimethyl sulfoxide (DMSO vehicle for nobiletin and AKT Inhibitors dilution (CDMSO)). Total cell number were determined based on Hoechst nuclei staining (left image), TE cells were detected by antiCDX2 (middle image) and DAPI positive CDX2 negative cells correspond to ICM (right image). Scale bar 50 µm.

4.3.4. Nobiletin supplementation modulates the AKT signaling pathway in early bovine embryos during EGA

Our results revealed that nobiletin ameliorates the negative effects of AKT inhibition on preimplantation development and embryo quality parameters in bovine.

Therefore, to determine whether nobiletin recovers the effects of AKT inhibition through modulation of rpS6 phosphorylation, one of the protein downstream of AKT in PI3K/AKT pathway activation, a western blot analysis showed that p-rpS6 level on 8-cell stage embryos from MNEGA was significantly lower from both AKT inhibitor groups compared to Nobiletin and control groups. When we supplemented the embryos with nobiletin in addition to the Inh III, nobiletin was able to partially recover the adverse effect of the inhibitor, showing a rpS6 phosphorylation level significantly higher ($P<0.05$), although the nobiletin was not able to recover totally the previous phosphorylation level showed in Nob and both control groups. In contrast, nobiletin was not able to reverse the effect caused by the Inh IV on the phosphorylation level of the rpS6 protein (Figure 31A).

In the case of 16-cell stage embryos from MJEGA, the presence of both AKT inhibitors decreased the p-rpS6 level significantly but, in this case, the supplementation with nobiletin was able to recover significantly ($P<0.05$) the p-rpS6 level in the presence of both Inh III and Inh IV. Although nobiletin was not able to recover totally the previous phosphorylation level showed in Nob and both control groups, the nobiletin, by itself, was able to generate a significantly higher level of p-rpS6 than any studied group level (Figure 31B).

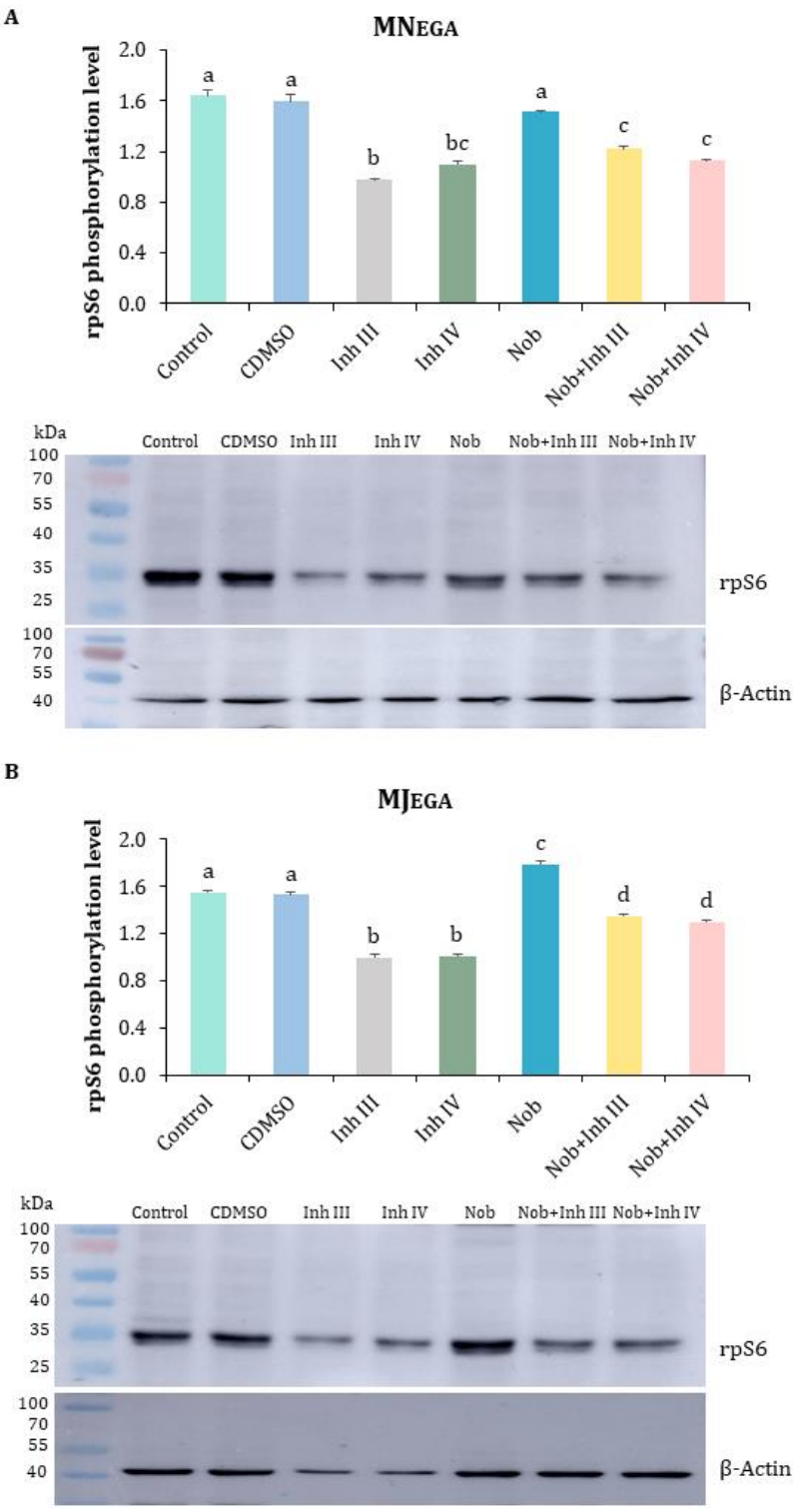

Figure 31. Representative Western blot images of rpS6 and actin protein levels in *in vitro* produced bovine early embryos. (A) 8-cell stage embryos cultured during 21-54 hpi (MNEGA: presumptive zygotes to 8-cell stage) and (B) 16-cell stage embryos cultured during 54-96 hpi (MJEGA: 8- to 16-cell stage) in SOF with 5% FCS (Control); supplemented or not with 15 µM of AKT Inhibitor III (Inh III) or with 10 µM of AKT IV Inhibitor (Inh IV), or with 10 µM nobiletin (Nob), or with 10 µM nobiletin + 15 µM of AKT Inhibitor III (Nob+Inh III), or with 10 µM nobiletin + 10 µM of AKT Inhibitor IV (Nob+Inh IV), or with 0.03% dimethyl sulfoxide (DMSO vehicle for nobiletin and AKT Inhibitors dilution (CDMSO)). Data were normalized relative to the abundance of actin and p-rps6 level. Data are the mean ± s.e.m. Different letters indicate significant difference ($P<0.05$) between treatments.

DISCUSSION

5. Discussion

Nobiletin, a class of polymethoxylated flavonoid identified from the citrus peel, is easily absorbed across the cytoplasmic membranes due to its structure and lipophilic nature. Besides, clear evidence showed that it interacts with several signaling pathways (ERK, PI3K/AKT, CREB) to promote survival in various cell lines and that it also has a broad range of biological effects including cell cycle regulation, reduction of apoptosis and antioxidation. To our knowledge, the present studies are the first to investigate the effects of nobiletin supplementation during IVM and IVC in oocyte quality and their developmental competence as well as embryo quality in cattle.

Nobiletin influences the steroidogenesis of cumulus cells during IVM

Cumulus cells play an important role during oocyte growth and maturation, they provide nutrients (Eppig, 1982) and mediate the effects of hormones during oocyte maturation (Zuelke & Bracketf, 1990). Mingoti *et al*. (2002) demonstrated that CCs of bovine COCs can secrete E_2 and P_4 in maturation media, and Endo *et al*. (2013) and Sakaguchi *et al*. (2018), demonstrated that exogenous and endogenous E_2 by GCs directly supports the *in vitro* development of bovine COCs. In the first experiment, supplementation with 25 and 50 μM nobiletin in maturation medium increases E_2 and P_4 production by CCs. This is in line with a study by Horigome *et al*. (2016) that demonstrated that nobiletin enhanced testosterone production in cultures of Leydig cells via cAMP/CREB signaling. Therefore, our results indicated that nobiletin might act directly or synergistically with other hormones during oocyte maturation to alter the CCs steroidogenesis *in vitro* and thus, the increase of P_4 and E_2 production, without any steroid hormone supplementation, plays a positive role in oocyte nuclear and cytoplasmic maturation.

Nobiletin promotes concomitant nuclear and cytoplasmic oocyte maturation

Nuclear maturation was improved by nobiletin supplementation to the IVM medium. This is in line with other studies using different antioxidants, such as resveratrol, astaxanthin or melatonin supplementation in bovine oocyte maturation *in vitro* (Pang *et al.,* 2018; Wang *et al.,* 2014). In contrast, other studies in pigs (Kere *et al.,* 2012) and cattle (Sovernigo *et al.,* 2017) using a broad spectrum of antioxidants did not show an effect on the nuclear maturation rate. These contradictory results suggest that different effects of antioxidants on nuclear maturation could be related to their capacity to activate the MAPK pathway since in mammals MAPK is responsible for meiotic progression (Fan & Sun, 2004), and in bovine oocytes the two main isoforms (ERK1/2) of MAPK are activated near the time of GVBD (Quetglas *et al.,* 2010). One of the biological effects of nobiletin is the activation of MAPK activity shown in different cell types (Huang *et al.,* 2016).

Hence, a more plausible explanation for an increase in M-II following nobiletin supplementation could be through P_4 and stimulation of MAPK1/ERK2, which plays a fundamental role in the regulation of microtubule organization, spindle assembly, chromosome distribution and meiosis resumption (Gordo *et al.,* 2001). A similar function was demonstrated for resveratrol with improved meiosis resumption by enhancing the expression of Mos/MEK1/p42 MAPK cascade genes (Wang *et al.,* 2014). Based on the above, it could be hypothesized that nobiletin regulates the secretion of androgens in the CCs, and improved meiosis resumption by activation of MAPK; however, further experiments are necessary to corroborate this hypothesis.

Next, we observed that supplementation of nobiletin during *in vitro* maturation improved also cytoplasmic maturation. The migration of CG to the cortical region of the oocyte, as well as mitochondrial distribution and their activity, are suitable indicators to analyze cytoplasmic maturation (El-Raey *et al.,* 2011; Mo *et al.,* 2014). Hosoe & Shioya, (1997) and Hoodbhoy *et al*. (2001) demonstrated that proteins released by the CG are also necessary for preimplantation embryo development. Our results demonstrated that the addition of 25 or 50 μM nobiletin to IVM medium significantly increased peripheral distribution of CG, suggesting that nobiletin at these concentrations could act by promoting a better organization of microfilaments and therefore, improving CG migration. A similar effect of CG migration was described with sodium nitroprusside for bovine oocyte *in vitro* maturation (Viana *et al.,* 2007).

Mitochondria play an important role since they are a key component of the metabolic machinery responsible for the supply of energy that is consumed during the maturation process (Ferreira *et al.,* 2009) and are also the main generator of free radicals in mammals (Brand *et al.,* 2013). The movement of mitochondria to areas of high energy consumption is crucial for the oocyte and the embryo during critical periods of the cell cycle. For this reason, the mitochondrial cytoplasmic distribution pattern has been associated with the quality and developmental capacity of mammalian oocytes and embryos (Egerszegi *et al.,* 2010). In the first experiment, we demonstrated that the addition of 25 or 50 μM nobiletin to IVM medium significantly increased mitochondrial migration, giving rise to granular aggregations throughout the cytoplasm in the oocyte after IVM. This pattern of distribution is similar to that described for bovine oocytes in other studies, which revealed that mitochondrial reorganization is necessary for cytoplasmic maturation, rearrangement of the cytoskeleton and developmental capacity after IVF (Lima *et al.,* 2018; Zhao *et al.,* 2018). Another important function of mitochondria is to synthesize ATP through β-oxidation and this process involves the electron transport chain (Ambrogi *et al.,* 2017). However, electrons may be lost during this process and could be bond to O_2, resulting in the production of ROS that decreases the developmental competence of the oocyte (Barja, 2007).

Thus, our results of increased oocyte mitochondrial activity could be related to the cytoprotective effects of nobiletin and its intrinsic ROS-scavenging property.

Nobiletin protects the oocyte from oxidative stress damage

Under normal conditions, cells maintain their ROS levels in equilibrium (Sovernigo *et al.,* 2017), while during IVM, the cells may suffer disturbances in redox equilibrium having deleterious effects on development (Pamplona & Costantini, 2011; Rocha-Frigoni *et al.,* 2016). However, studies demonstrated that the addition of antioxidants into the culture medium reduces the harmful effects of ROS during IVM and offers a way of protecting the oocyte and subsequent embryo (Kere *et al.,* 2012; Sovernigo *et al.,* 2017). In the oocyte, the main ROS scavenger system GSH uses a reducing power provided by oxidative metabolism (Rocha-Frigoni *et al.,* 2016). Our results show that 25 or 50 μM nobiletin supplementation in IVM medium reduced the intracellular ROS levels, which is in agreement with the use of other flavonoids such as resveratrol (Wang *et al.,* 2014), quercetin and taxifolin (Kang *et al.,* 2016) and other classes of antioxidants like vitamin C (Sovernigo *et al.,* 2017). Regarding nobiletin, studies in cell cultures demonstrated its ability to significantly decrease ROS generation (Choi *et al.,* 2007) but to date, there are no studies available on its effects in oocytes and embryos. Nobiletin has a beneficial effect on cell protection (Huang *et al.,* 2016), and like other antioxidants, this effect could be produced due to its hydrophobic nature, which allows it to incorporate into the membrane (Kang *et al.,* 2016), inhibiting ROS attack and decreasing lipid peroxidation. Therefore, the positive effect observed in our study could be attributed to this property.

In cytoplasmic maturation, GSH is considered a biochemical marker for oocyte quality, and plays an important role in maintaining redox homeostasis, hence protecting the embryo from oxidative damage before genomic activation (de Matos & Furnus, 2000). Our results showed decreased levels of GSH in 25 or 50 μM nobiletin supplemented groups, opposed to other studies reporting either a reduction in ROS levels associated with an increase in GSH levels (Sovernigo *et al.,* 2017; Wang *et al.,* 2014) or no increase in GSH levels in bovine oocytes (Rocha-Frigoni *et al.,* 2016). A reasonable explanation for this could be due to the highest mitochondrial activity found in the oocytes from Nob25 and Nob50 groups. It is widely known that increased mitochondrial activity leads to an increase in the exchange of electrons in the inner mitochondrial membrane, which is considered one of the main sources of ROS production (Zhao *et al.,* 2011). Despite a high mitochondrial activity, the intracellular ROS levels in the mature oocytes from the Nob25 and Nob50 groups were lower than those observed in the oocytes from the control groups, suggesting that GSH was consumed to avoid the harmful effects of the high levels of ROS. This explanation has been proposed before by Rocha-Frigoni *et al*. (2016) for cysteine and cysteamine antioxidant

activity during bovine oocyte IVM and by Qu *et al*. (2018) for nobiletin reduction of ROS levels in response to cadmium-induced neuronal injury in rats.

Nobiletin improves oocyte developmental competence and embryo quality

Improvements in oocyte quality by 25 and 50 μM nobiletin supplementation during *in vitro* maturation were reflected by increased blastocyst development rates on Day 7 and 8. These results are in line with other studies which evaluated other flavonoids like resveratrol *(Wang et al.,* 2014), or antioxidants such as cysteamine (Sovernigo *et al.,* 2017), vitamin C (Torres *et al.,* 2019), lycopene (Chowdhury *et al.,* 2017) and carnitine (Mishra *et al.,* 2016) in the IVM medium. Furthermore, flavonoids or antioxidants in the IVM showed an interaction with the expression of certain qualitatively related genes to the development of mature oocytes and/or the production of blastocysts.

To test whether the effects of nobiletin during IVM were related to gene expression changes, we analyzed the expression of candidate genes for oxidative stress, embryo development, and quality. *SOD2*, an indicator of oxidative stress (Mishra *et al.,* 2016) was downregulated in oocytes and CCs obtained from Nob25 and Nob50 groups compared with controls, whereas in blastocysts it was not altered. This is in accordance with the findings of Gülçin (2006), who showed that superoxide plays an important role in the neutralization of ROS, so a reduction in ROS formation requires less *SOD2* to neutralize free radicals. On the other hand, *CLIC1* is considered a sensor of cell oxidation (Averaimo *et al.,* 2010; Goodchild *et al.,* 2009) and is involved in ROS production (Averaimo *et al.,* 2010). Our results showed that *CLIC1* was downregulated in blastocysts obtained from Nob25 and Nob50 groups compared with the controls, in both cases with increased embryo yield, which agree with earlier studies showing that *CLIC1* expression accompanied by low accumulation of ROS improves embryo development (Hamdi *et al.,* 2018). These findings together with the low intracellular ROS and GSH levels in the oocytes matured with nobiletin supplementation indicate an improvement of their antioxidant activity and consequently an enhanced quality of the produced blastocysts.

On the other hand, *CYP51A1*, *BMP15*, *MAPK1*, *GJA1* and *BCL2*, are genes considered quality biomarkers of *in vitro* matured oocytes (Li *et al.,* 2015; Salhab *et al.,* 2013). *CYP51A1* participates in the regulation of cholesterol biosynthesis (Nakamura *et al.,* 2015) and it has been demonstrated that biosynthesis of cholesterol is one example of metabolic cooperation between GCs and oocytes (Su *et al.,* 2009). Furthermore, the upregulation of the enzyme coded by *CYP51A1* is a result of negative feedback reflecting lowered cholesterol availability, which is implied in the lower quality of oocytes (Nakamura *et al.,* 2015). Therefore, downregulation of *CYP51A1* mRNA

expression observed in oocytes and their CCs matured with nobiletin supplementation could be an indicator of good quality. In contrast, 50 μM nobiletin supplementation in IVM upregulated the expression of *CYP51A1* in blastocysts. This is in line with the results of nobiletin supplementation in liver cell culture (HepG2), showing upregulation of CYP1 (Cytochrome P450s family) and improved cholesterol synthesis due to full methoxylation in the A-ring of nobiletin chemical structure (Huang *et al.,* 2016; Noguchi *et al.,* 2016). Hence, nobiletin could act differently depending on the cell type, probably due to the bioactivity or its chemical structure, which causes that *CYP51A1* might be down or upregulated to control cholesterol availability, however, more in-depth studies are necessary to corroborate this information.

In mammals, *BMP15* is known to be involved in oocyte maturation and cholesterol biosynthesis, being specifically expressed in oocytes and acting on CCs, improving oocyte competence, and early embryo development in cattle (Caixeta *et al.,* 2013; Hussein *et al.,* 2011). Several studies reported an increase in *BMP15* transcript during maturation in buffalo (Kathirvel *et al.,* 2013) and dog (Lee *et al.*, 2017) oocytes, which are consistent with our findings of an increase in *BMP15* expression in oocytes and their CCs matured with nobiletin supplementation in IVM, related with their improved developmental competence. The MAPK family plays an important role in bovine oocyte maturation by inducing GVBD (Inoue *et al.,* 1998). Likewise, *MAPK1* mRNA plays a key role in oocyte maturation by acting on granulosa and CCs in various species including cattle (Quetglas *et al.,* 2010) and dogs (Lee *et al.,* 2017). Our results demonstrated that *MAPK1* mRNA expression in oocytes and embryos was upregulated, suggesting that nobiletin could act on cell cycle regulation as reported by Yoshimizu *et al*. (2004) and Morley *et al*. (2007) in other types of cells. On the other hand, *GJA1,* also known as connexin 43 (Cx43), is a component of gap junctions expressed in CCs and a major mediator of cell-to-cell communication via gap junctions, and a proliferation regulator (Brązert *et al.,* 2020). Recently, it was shown that CCs of bovine oocytes with higher developmental competence express higher *GJA1* (Read *et al.,* 2018). These findings are in agreement with our results demonstrating higher *GJA1* expression in the CCs from oocytes matured with nobiletin. Taken together, these results suggest that nobiletin modifies the expression of key genes for oocyte cytoplasmic development and maturation, improving their developmental competence and increasing embryo yield.

Moreover, we observed that during IVM, nobiletin decreased the expression of *BCL2* in CCs. The downregulation of *BCL2* expression is associated with a protective effect and has been reported to have a critical role in CCs by acting as a regulator of apoptosis (Boelhauve *et al.,* 2005). Studies in cattle showed that lycopene (antioxidant) supplementation during *in vitro* maturation, increases expression of *BCL2* exerting a pro-apoptotic effect (Chowdhury *et al.,* 2017). Studies that used nobiletin on human cancer cell lines (gastric, hepatic, and breast) showed that nobiletin

induced apoptotic cell death by reducing the expression of *BCL2* (Chen *et al.,* 2014; Huang *et al.,* 2016; Morley *et al.,* 2007). However, the molecular mechanisms whereby nobiletin induces apoptosis among different carcinogenic cells remain poorly understood. Therefore, it is to be assumed that nobiletin has different actions for healthy and unhealthy cells or depending cell types.

Nobiletin supplementation during EGA improves the development and quality of preimplantational embryos.

Irrespective of concentration, addition of nobiletin to culture media during MNEGA phase (21-54 hpi) did not affect cleavage rates at 54 hpi as well as the percentage of embryos reach the 8-cell stage but increased blastocyst production, whereas nobiletin supplementation in culture media during MJEGA phase (54-96 hpi) significantly increased the percentage of embryos that reach the16-cell stage and blastocyst production. Several studies have shown that during EGA the bovine embryo actively synthesize transcription factors and this process directly links to chromatin changes, protein allocation, nuclear reorganization and cell proliferation (Graf *et al.,* 2014; Lequarre *et al.,* 2003). Since in our results developmental kinetics were stimulated with more 16-cell embryos by nobiletin during MJEGA, we could hypothesize that nobiletin activates early embryonic genes important for the proper genomic function of the embryo during major EGA. Although with our experimental design we cannot link this effect specifically with either of the two activation phases of the embryonic genome.

The evidence that nobiletin supplementation improves blastocyst production is in line with other studies showing increased embryo development *in vitro* when culture medium was supplemented with biological antioxidants similar to nobiletin (Chowdhury *et al.,* 2018; dos Santos *et al.,* 2019; Zullo *et al.,* 2016a,b). Another effect of nobiletin was to induce a significant increase in mitochondrial activity and a lower content of lipid droplets in blastocysts from both EGA phases analyzed. Mitochondria, so, are considered as energy control units necessary for cell division, pluripotency and differentiation (Ge *et al.,* 2012). Cagnone & Sirard, (2016) reported that *in vivo,* during the early cleavages, mitochondria and intracellular metabolism are quiescent. Nevertheless, *in vitro*, this metabolic quiescence is altered due to the presence of nutrients in excessive amounts that overstimulate mitochondria and alter their efficiency to respond to oxidative phosphorylation (Cagnone & Sirard, 2016). Mitochondria also sense changes in redox potential and force embryos to adapt versus the decreased production of ATP by oxidative phosphorylation during the transition from morula to blastocyst (Cagnone & Sirard, 2016; Ge *et al.,* 2012). Besides, some studies reported that changes in mitochondrial activity may affect the development of energetic metabolism in the embryo, in terms of availability of glucose, lipids,

amino acids and DNA methylation (Cagnone & Sirard, 2016; Harvey, 2019). Although the nobiletin action mechanism in mitochondria has not been fully elucidated, we previously observed that increased oocyte mitochondrial activity was related to the cytoprotective effects of nobiletin and its intrinsic ROS-scavenging property (Cajas *et al.,* 2020). Nevertheless, the effect in the blastocyst could be explained based on the fact that nobiletin is a hydrophobic compound, which easily penetrates through cell membranes directly affecting mitochondrial bioenergetics. Nobiletin can modify intramitochondrial proteins (e.g. acetylated proteins localized within the mitochondria in the brain of rats) (Jojua *et al.,* 2015) or alter the mitochondrial membrane potential by changing the activities of mitochondrial enzymes, like succinate dehydrogenase and cytochrome c oxidase as it has been demonstrated in human blood lymphocytes (Dabrundashvili *et al.,* 2011).

Lipid content is a crucial factor for early embryo development *in vitro* in bovine since energy metabolism is abnormal under such conditions, resulting in an excessive accumulation of lipids associated with reduced embryonic quality (Ghanem *et al.,* 2014). Lipids are stored in intracellular droplets and are metabolized via β-oxidation in the mitochondrial matrix. A large amount of lipid droplets increases the production of ATP necessary for the formation of blastocysts but this can affect its quality; thus, a lower number of lipid droplets in blastocysts is considered as a criterion of good quality embryos (Cañón-Beltrán *et al.,* 2020; dos Santos *et al.,* 2019). Our results showed for both EGA phases, nobiletin supplementation in culture medium reduced the amount of lipids in blastocysts. Furthermore, we analyze the expression of proliferator-activated receptor alpha transcript (*PPARα*), belonging to one of the 3 key nuclear receptors in the modulation of transcription for lipid metabolism-related genes (Wahli & Michalik, 2012). *PPARα* was previously detected in cattle embryos and its expression has been associated with embryo quality (Childs *et al.,* 2008). In our study, *PPARα* was significantly upregulated in blastocysts produced with both concentrations of nobiletin supplementation during MNEGA phase or 10 µM of nobiletin supplementation during MJEGA compared to controls. These results together are in line with other studies which demonstrated that antioxidant supplementation in IVC medium, like crocetin (Zullo *et al.,* 2016b) and L-carnitine (Mishra *et al.,* 2016), improved embryo quality by decreasing their lipid content. Regarding nobiletin, studies in mice showed its ability to reduce hepatic lipid accumulation, prevent lipoprotein overproduction and normalize insulin sensitivity when supplied in the diet (Mulvihill *et al.,* 2011). Moreover, it has been demonstrated that nobiletin reduces lipid accumulation and regulates lipidic metabolism in hepatic cell lines (Huang *et al.,* 2016; Lin *et al.,* 2011). There is evidence that nobiletin upregulates the expression of *PPARα* in white adipose tissue of mice (Huang *et al.*, 2016). An explanation for the reduction of lipids by nobiletin has been proposed indicating that

full methoxylation of the A-ring of nobiletin seems to be the most optimal structure to express potent effects on modulating hepatic lipid metabolism via primarily suppressing lipoprotein secretion in HepG2 cells (Lin *et al.,* 2011). Therefore, it appears that the ability of nobiletin to reduce lipid content and improve mitochondrial activity in blastocysts may be related to the properties of its chemical structure that allows modulation of lipid metabolism and mitochondrial activity. Moreover, activation of *PPARα* by nobiletin could result in increased embryo lipid turnover through the β-oxidative pathway, preventing accumulation of lipoperoxides despite peroxisomal induction. Recent studies have shown that response of embryos to IVC involves a variety of metabolic factors that act as signals of extracellular and intracellular conditions to which the early embryos can adapt cell programming, signaling pathways, mitochondrial metabolism (mitochondrial production of Acetyl-Coenzyme A (Acetyl-Co A) and methyl groups, which are dependent on the availability of glucose, lipids and amino acids) or peroxisome proliferator-activated receptors (PPARs) in response to lipid content. These factors in the embryo are translated into effects on developmental speed or epigenetic modifications (Cagnone & Sirard, 2016; Canovas *et al.,* 2017; Ramos-Ibeas *et al.,* 2019). Consequently, this mechanism can reinforce the antioxidant-defense role of nobiletin during the early embryo development *in vitro*.

Embryo cell number is another parameter correlated with embryonic development and quality. Also, it has been reported for different cellular lines (MOLT-4, HUVEC, PC12D, K-N-SH cells) that nobiletin exerts its activity by modulating cell cycle progression (Huang *et al.,* 2016). We observed that regardless of EGA phase (MNEGA and MJEGA), nobiletin supplementation in culture media increased the total cell number of produced blastocysts. This increase rate was similar to that observed with other antioxidants such as vitamin C (Hu *et al.,* 2012) or crocetin (dos Santos *et al.,* 2019; Zullo *et al.,* 2016b), suggesting that nobiletin could directly stimulate the cell cycle during EGA and therefore, improve embryo quality.

To verify whether if the effects of nobiletin during MNEGA or MJEGA were related to gene expression changes in the produced blastocysts, we analyzed the expression of genes related to oxidative stress during early embryo development. *GPX1*, considered the major antioxidant enzyme within the Glutathione peroxidase family, is ubiquitously expressed in the cytosol and also has been found in mitochondria (Ufer & Wang, 2011). *GPX1* acts as a scavenger of hydrophilic peroxide species, can be transformed into an enzymatically inactive cellular structural component, and protects cells against oxidative damage (Ufer & Wang, 2011). During *in vitro* production ROS generation increases and one of the defenses to counter excess ROS in the embryo is *GPX1*; therefore, *GPX1* overexpression has been positively linked with embryo quality (Cebrian-Serrano *et al.,* 2013; He *et al.,* 2017). In our study, gene expression analysis revealed the upregulation of *GPX1* in 8- and 16-cell embryos as well as in blastocysts produced with nobiletin

supplementation during MNEGA or MJEGA phases. A similar response has been reported in sheep and bovine embryos treated with other types of antioxidants like L-carnitine (Mishra *et al.,* 2016) or crocetin (dos Santos *et al.,* 2019). *NFE2L2* transcript (also known as *Nrf2*) is important for embryo tolerance to oxidative stress during EGA as well as for its competence for development (Gad *et al.,* 2012). PI3K/AKT pathway plays a role in regulating *NFE2L2* activation and is involved in the regulation of protein kinases, which may induce nuclear translocation (Zou *et al.,* 2013). Harris & Hansen, (2012) and Ghanem *et al*. (2014) reported in mice that up-regulation of *NFE2L2* transcript may protect embryos from oxidative stress through preservation of intracellular redox states to ensure normal embryonic development. In the same line, our results showed the relative abundance of *NFE2L2* transcript increased in 16-cell stage embryos cultured with 10 µM nobiletin during MJEGA, while remained unaltered in 8-cell stage embryos as well as in blastocysts from both treatments, indicating an embryo stage and EGA phase -dependent effect. Moreover, data obtained in cancer cells of mice showed that *NFE2L2* mRNA levels were upregulated when nobiletin was supplemented in culture medium (Wu *et al.,* 2017). Taken together, these data indicate that nobiletin plays an antioxidant-defense role via distinct pathways during the different phases of early embryo development *in vitro*.

On the other hand, *CDK2* is necessary for cell cycle progression, and is a major kinase that governs AKT phosphorylation (Liu *et al.,* 2014), and participates in EGA (Milazzotto *et al.,* 2012). In our study, *CDK2* mRNA expression was upregulated in 8-cell (MNEGA), 16-cell and blastocysts (MJEGA) cultured with nobiletin. This is in line with Milazzotto *et al*. (2012) study in bovine embryos, that showed changes in the levels of transcription in genes associated with cell cycle and observed an increase in *CDK2* expression during early embryo development (8 and 16-cell embryos, and blastocysts). Conversely, studies using nobiletin in cancer cells (U87, Hs683) showed a decrease in *CDK2* expression (Huang *et al.,* 2016; Lien *et al.,* 2016). Hence, nobiletin seems to respond differently depending on the cell type.

Histone H3.3 is encoded by *H3-3A* and H*3-3B* genes and is related to DNA synthesis and integrated into embryonic nucleosomes to mark genes for subsequent expression in development (Jang *et al.,* 2015). We observed that supplementation of 5 or 10 µM nobiletin during MNEGA, increased the expression of *H3-3B* and *H3-3A* genes in 8- cell embryos while supplementation of 10 µM nobiletin during MJEGA increased the expression of *H3-3B* gene in 16-cell embryos. This is in corroboration with results from a recent study were characterization of the expression of both genes that encode *H3.3* (*H3-3A* and *H3-3B*) was performed in early bovine embryos, demonstrating that *H3-3B* mRNA is very abundant throughout early embryogenesis, being two to three times higher than *H3-3A* mRNA during the major wave of EGA (Zhang *et al.,* 2018a). Additionally, a higher abundance of *H3-3B* compared to *H3-3A* was found in mouse embryos (Wen

et al., 2014), suggesting that the protein encoded by *H3-3B* gene may be critical for initiating the transcription of embryonic genes during EGA. As mentioned above, EGA is crucial for further embryo development and regulated by several important factors (Lee *et al.,* 2013). One crucial factor is histone modification, including methylation and acetylation (Lindeman *et al.,* 2011). Likewise, Acetyl-Co A is a central metabolite linking glucose oxidation and long-chain fatty acid or cholesterol synthesis, providing energy and materials for cell growth and proliferation. Furthermore, Acetyl-Co A, as a donor of an acetyl group, can be utilized by histone acetyltransferases for histone acetylation (Sivanand *et al.,* 2018). A recent study showed that Acetyl-CoA synthases are essential for maintaining histone acetylation under metabolic stress during EGA in pigs and they corroborated that β-oxidation is crucial for porcine embryo development by contributing to energy metabolism and histone acetylation (Zhou *et al.,* 2021). This suggests one more time that nobiletin could prefer the β-oxidation pathway as an energy production mechanism.

Nobiletin increases AKT phosphorylation in blastocysts

During *in vitro* development, embryos have a series of metabolic factors that are required in proliferation, differentiation, and survival of cells (Manning & Toker, 2017; Plotnikov *et al.,* 2011). In this context, the quality of the embryos produced *in vitro* depends on many factors, among them the expression of different genes, which depends on different signaling pathways that play important roles in the formation of the blastocyst; being one of them PI3K/AKT (Manning & Toker, 2017; Plotnikov *et al.,* 2011; Zhang *et al.,* 2007, Cecconi *et al.,* 2012). AKT regulates cellular processes such as glucose metabolism, transcription, cellular growth and proliferation (Manning & Toker, 2017). In blastocysts, AKT inhibition arrest their development and AKT activation triggers the differentiation and migration of TE cells (Ashry *et al.,* 2018; Marques & Thorsteinsdóttir, 2013). Other studies showed that AKT appear to have an important role in early embryonic development, in double-knockout mice deletion of any of the AKT isoforms leads to morphological abnormalities or even exhibit a more severe phenotype related to early lethality (Dummler *et al.,* 2006). Moreover, PI3K/AKT regulates the development of preimplantation embryo by mediating the effects of autocrine factors (O'Neill, 2008). Previous studies in cell lines have shown that nobiletin can act through various signaling pathways, including AKT (Huang *et al.,* 2016). However, as far as we know, nobiletin action on AKT pathway in bovine blastocysts produced *in vitro* is unknown. In experiment 2 we established the presence of the AKT pathway in bovine blastocysts. Expression of the AKT protein and its phosphorylation status were confirmed by western blot analysis of bovine blastocysts produced with or without nobiletin supplementation during MNEGA or MJEGA phase. Similar results were found by Ashry *et al*. (2018), who investigated the relationship between AKT signaling and the embryotrophic

actions of follistatin, indicated that it plays an important role in the regulation of AKT signaling in early bovine embryos. Together, these results suggest that nobiletin is associated with increased AKT phosphorylation and, as it has been shown in cell lines studies, nobiletin has the ability to interact with this pathway, and regulate specific genes. Accordingly, our results showed increased AKT phosphorylation that might be related to the upregulation of genes that favor the progression of the cell cycle (*CDK2*) and embryo quality (*PPARα*).

Nobiletin-induces partial abrogation of AKT inhibition deleterious effects on preimplantation bovine embryo development *in vitro*

Taking into account that nobiletin increases phosphorylation of AKT, in experiment 3 we used two different inhibitors of AKT activity (Inhibitor III and inhibitor IV) to reduce the bovine embryo developmental capacity during EGA (MNEGA or MJEGA) and to investigate if nobiletin could interact through that signaling pathway. Our results corroborate that the kinetics of early embryo development as well as blastocysts yield from both phases of EGA treatments with AKT inhibitors were significantly reduced whereas supplementation of 10 μM nobiletin enhances them. However, the most interesting result was to see how this AKT inhibitory effect was reversed when nobiletin was combined. A similar effect was reported when follistatin was added to the culture medium in bovine embryos treated with AKT inhibitors, countering the inhibitor effect by improving the embryonic development and production rates (Ashry *et al.*, 2018). Previously, it was reported that PI3K inhibition, upstream kinase of AKT, result in a significant decrease of total cleavage, 8-cells and blastocyst rates in bovine (Aparicio *et al.,* 2010). Moreover, AKT is implicated in regulation of mitotic cell cycle transition from G2 to M phase through activation of MPF (Manning & Cantley, 2007); thus, if AKT is inhibited, the MPF complex remains inactive and cells are arrested in G1 or G2 stages of the cell cycle (Baran *et al.,* 2013).

To verify the nobiletin action in combination or not with AKT inhibitors during MNEGA or MJEGA on the quality of 8- and 16-cell stage embryos, respectively and its possible implication in pathways signaling, we analyzed the expression of the genes previously selected for oxidative stress (*GPX1* and *NFE2L2).* Our results showed that the relative abundance of *GPX1* and *NFE2L2* transcripts in 8- (MNEGA) and 16-cell (MJEGA) stage embryos was decreased for both AKT inhibitor groups, while it was increased for Nob group. Nevertheless, nobiletin supplementation to the AKT inhibitor groups partially recovered the expression. During *in vitro* production, *GPX1* overexpression is positively related to embryo quality (Cebrian-Serrano *et al.,* 2013; He *et al.,* 2017) and upregulation of *NFE2L2* transcript preserve the embryo from an intracellular redox state (Ghanem *et al.,* 2014; Harris & Hansen, 2012). Research in many type of cells have reported that PI3K/AKT and MAPK/ERK pathways are involved in the expression of *NFE2L2* (Gao *et al.,*

2013; Nguyen *et al.,* 2013). In PC12 cells nobiletin enhanced the binding of *NRF2* to the antioxidant response element for gene expression. This cytoprotective effect of nobiletin was mediated by modulation of PI3K/AKT signaling (Li *et al.,* 2014; Su *et al.,* 2012). In HCT8/T cells nobiletin interacts with the ERK/NRF2 signaling pathway and significantly reduces its phosphorylation (Feng *et al.,* 2020).

POU5F1 is exclusively expressed in ICM after blastocoel formation (Wu & Schöler, 2014) and is crucial for normal epiblast differentiation (Niwa *et al.,* 2005), whilst *CDX2* is essential for establishing the trophectoderm in bovine embryos (Goissis & Cibelli, 2014; Sakurai *et al.,* 2016). Our results revealed downregulation of *POU5F1* gene in 8- (MNEGA) and 16-cell (MJEGA) stage embryos as well as of *CDX2* gene in 16-cell (MJEGA) stage embryos produced with AKT inhibitors, while an upregulation was revealed for nobiletin groups. However, nobiletin in presence of AKT inhibitors was able to recover partially the expression of these genes. It has been evidenced that *POU5F1* transcript is present in the bovine embryo during the 8- to 16-cell stages, when major EGA occurs (Graf *et al.,* 2014; Kuijk *et al.,* 2012). Also, it has been shown that the transcription level of *POU5F1* is higher in competent embryos and together with *CDX2* are essential for early development and differentiation of ICM and TE in bovine embryos (Simmet *et al.,* 2018). Ashry *et al.* (2020), demonstrated that *CDX2* is a molecular determinant of blastocyst quality and conceptus elongation in bovine. Thus, embryos that express low *POU5F1* and *CDX2* levels may be considered of inferior quality (Moussa *et al.,* 2019). Moreover, it has been reported that expression of *CDX2* and *POU5F1* can be regulated by AKT and ERK pathways (Mnatsakanyan *et al.,* 2019; Xu *et al.,* 2019).

In corroboration with the above, we evidenced that the number of total, TE and ICM cells from blastocysts produced with nobiletin during MNEGA or MJEGA were significantly higher than them from AKT inhibitors. Although, nobiletin supplementation to AKT inhibitors groups rescue their negative effect by increasing the number of total, TE and ICM cells in blastocyst. It has been demonstrated that embryo quality could be defined by blastocyst total cell number and their differentiation to ICM and TE, while the ICM/TE ratio is an important index of embryo survival and pregnancy rate (Fleming *et al.,* 2004). In addition, the TE cells have a fundamental role establishing a pregnancy around Day 14 in bovine, when intense trophoblastic proliferation begins together with increased trophoblastic secretion of the pregnancy recognition factor interferon-tau (Ealy & Yang, 2009; Sakurai *et al.,* 2016). Therefore, these results together with the expression level of *POU5F1* and *CDX2* transcripts revealed the critical role of AKT at early stages of preimplantation embryo development and could open the possibility of establishing ERK as an important pathway of action for nobiletin in bovine embryo.

FOSL1 is another gene related to development of the trophoblast lineage and placentation (Lee *et al.,* 2018). We found a downregulation of the expression of *FOSL1* gene in 16-cell stage embryos (MJEGA) produced with AKT inhibitors, while it was upregulated in 16-cell stage embryos from nobiletin group. Nevertheless, as in all previously described transcripts, nobiletin supplementation in presence of AKT inhibitors showed a partially recovered expression. *FOSL1* acts as a downstream effector of the PI3K/AKT signaling pathway (Kent *et al.,* 2011). It has been showed that loss of PI3K/AKT signaling results in decreased *FOSL1* accumulation and disruption of PI3K/AKT with small-molecule inhibitors interferes with the differentiation of trophoblast gene expression profile (Kent *et al.,* 2011). Besides, Renaud *et al*. (2014) confirmed that *FOSL1* increases during trophoblast cell differentiation in a PI3K/AKT-dependent manner. In addition, studies in PC12 cells reported that nobiletin upregulates the mRNA expression of c-FOS and improves the activation of extracellular signal linked to the activation of AKT/ERK/MEK/CREB signaling pathways (Huang *et al.,* 2016; Kimura *et al.,* 2014a). Hence, these results could suggest that both ERK and PI3K/AKT pathways probably are involved in nobiletin regulation effects on embryo development.

In this sense, we analyzed the phosphorylation of the rpS6 protein, downstream of AKT, in *in vitro* produced bovine 8-cell (MNEGA) and 16-cell (MJEGA) stage embryos, and the p-rpS6 level was dramatically decreased upon AKT inhibitors treatment. Even so, nobiletin treatment not only reverses the AKT inhibitors effect on phosphorylation level but also increases the p-rpS6 on when was supplemented alone in MJEGA phase. These results suggest that nobiletin may be playing a role in returning the p-rpS6 to the levels required for rescuing the adverse effect of AKT inhibitors during EGA. One of the most important functions of rpS6 is that its phosphorylation is necessary for efficient global protein synthesis (Meyuhas, 2015). Previous reports have established that lack of rpS6 affects cell cycle progression: in mouse embryonic cells, where rpS6 was inhibited, embryos became apoptotic on post-embryonic day 5.5 and led to perigastrulation lethality (Panić *et al.,* 2006). Recently, in a study of porcine TE and uterine luminal epithelial cells, it has also been reported that an increase in the phosphorylation of AKT induces the activation of the rpS6 and the use of inhibitors of AKT activation abolished p-rpS6 (Jeong *et al.,* 2017).

Even so, if when AKT is inhibited and therefore with a decrease of the amount of p-rpS6, the presence of nobiletin increases this phosphorylation and that must come from another signaling pathway different from PI3K/AKT. In that sense, some studies have shown that rpS6 can be activated by 1) PIP3/AKT/TSC/Rheb/TORC1 pathway that conveys signals to S6Ks, which phosphorylates rpS6, 2) the activation of S6Ks by direct phosphorylation of PDK1 in a reaction that does not need binding of PDK1 to PIP3 and, 3) the activation of the second family of rpS6 kinases: Ras/Raf/MEK/ERK/RSK that inhibits the TSC1-TSC2 complex and, therefore, increases

signaling of TORC1 towards S6K (Magnuson *et al.,* 2011; Meyuhas, 2015; Ruvinsky & Meyuhas, 2006). These pathways share the same initial event, that is the binding of the ligand, but without the need for AKT participation. Hence, these pathways exert a parallel effect on both p-rpS6 and the activity of the respective kinase, this leads us to consider the possibility that for rpS6 activation during embryonic development, nobiletin is not only interacting through PIP3/AKT pathway but also interacting with the previous mentioned pathways. Moreover, as has been shown in studies with cell line that nobiletin attenuated cancer cell metastasis by inhibiting adhesion, invasion and migration via modulation of both MAPK and PI3K/AKT pathways (Ashrafizadeh *et al.,* 2020; Huang *et al.,* 2016; Shi *et al.,* 2013).

Collectively, our results suggest that nobiletin promotes oocyte and embryo quality and stimulates bovine early embryonic development *in vitro*. Besides, nobiletin supplementation in presence of AKT inhibitors retrieve their adverse effects on developmental competence along with embryo quality by increasing the total, TE and ICM cells and modifying the relative abundance of keys genes linked to cell proliferation and differentiation. Moreover, nobiletin increases the p-rpS6 level whether or not AKT inhibitors were present, which infers that nobiletin probably uses another signaling system in addition to MAPK cascade during oocyte maturation and PI3K/AKT pathway during early embryo development in bovine. Still, future studies are necessary to establish the specific receptors and pathways that nobiletin modulates during *in vitro* embryonic development.

CONCLUSIONS

Conclusions

1. Nobiletin can be used as a novel antioxidant to overcome ROS disorders in *in vitro* produced bovine embryos.
2. During *in vitro* maturation nobiletin supplementation improves oocyte nuclear and cytoplasmic maturation, decreases intracellular ROS and GSH levels, modifies the expression of genes linked to metabolism, development, and oxidative stress, as well as enhances oocyte developmental competence and embryo development and quality.
3. Nobiletin supplementation during *in vitro* culture (MNEGA or MJEGA phase) stimulates mitochondrial activity, as well as reduces the cytoplasmatic accumulation of lipids and promotes the expression of genes that regulate oxidative stress and lipid metabolism in key stages of bovine preimplantational embryo.
4. Beneficial effects of nobiletin supplementation during EGA on embryo development and quality of the produced blastocysts *in vitro* could be modulated by the activation of AKT signaling pathway.
5. Nobiletin supplementation in presence of AKT inhibitors during EGA retrieve their adverse effects on developmental competence along with embryo quality by increasing the total TE and ICM cells and modifying the relative abundance of keys genes linked to cell proliferation and differentiation.
6. Increase of p-rpS6 level by nobiletin, irrespective of AKT inhibitors, suggests the use of another signaling system than PI3K/AKT pathway during early embryo development in bovine.

CONCLUSIONES

Conclusiones

1. La nobiletina se puede utilizar como un nuevo antioxidante para paliar los trastornos de ROS en embriones bovinos producidos *in vitro*.
2. Durante la maduración *in vitro*, la suplementación con nobiletina mejora la maduración nuclear y citoplasmática de los ovocitos, disminuye los niveles intracelulares de ROS y GSH, modifica la expresión de genes vinculados al metabolismo, desarrollo y estrés oxidativo, así como mejora el desarrollo de los ovocitos y el desarrollo y la calidad del embrión.
3. La suplementación con nobiletina durante el cultivo *in vitro* (fase de MNEGA o de MJEGA) estimula la actividad mitocondrial de los blastocistos, reduce la acumulación citoplasmática de lípidos y promueve la expresión de genes relacionados con el estrés oxidativo y el metabolismo lipídico, en fases claves del desarrollo embrionario preimplantacional bovino.
4. Los efectos beneficiosos de la adición de nobiletina durante la EGA sobre el desarrollo embrionario y la calidad de los blastocistos producidos *in vitro* podría ser modulados mediante la activación de la vía de señalización de AKT.
5. La suplementación con nobiletina en presencia de inhibidores de AKT durante la EGA recupera los efectos adversos sobre la competencia del desarrollo junto con la calidad del embrión al incrementar el número de células totales, del TE y de la ICM y modificar la abundancia relativa de genes clave relacionados con la proliferación y diferenciación celular.
6. El aumento del nivel de p-rpS6 provocado por la nobiletina, independientemente de los inhibidores de AKT, sugiere el uso de otra ruta de señalización distinta a la vía PI3K/AKT durante el desarrollo embrionario temprano en bovinos.

BIBLIOGRAPHY

Abe, H., Yamashita, S., Satoh, T., & Hoshi, H. (2002). Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Molecular Reproduction and Development*, *61*(1), 57–66. https://doi.org/10.1002/mrd.1131

Adhikari, D., & Liu, K. (2014). The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes. *Molecular and Cellular Endocrinology*, *382*(1), 480–487. https://doi.org/10.1016/j.mce.2013.07.027

Agarwal, A., Gupta, S., Sekhon, L., & Shah, R. (2008). Redox Considerations in Female Reproductive Function and Assisted Reproduction: From Molecular Mechanisms to Health Implications. *Antioxidants & Redox Signaling*, *10*(8), 1375–1404. https://doi.org/10.1089/ars.2007.1964

Ambrogi, M., Dall'Acqua, P. C., Rocha-Frigoni, N. a. S., Leão, B. C. S., & Mingoti, G. Z. (2017). Transporting bovine oocytes in a medium supplemented with different macromolecules and antioxidants: Effects on nuclear and cytoplasmic maturation and embryonic development in vitro. *Reproduction in Domestic Animals*, *52*(3), 409–421. https://doi.org/10.1111/rda.12923

Aoki, K., Yokosuka, A., Mimaki, Y., Fukunaga, K., & Yamakuni, T. (2013). Nobiletin induces inhibitions of Ras activity and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase signaling to suppress cell proliferation in C6 rat glioma cells. *Biological & Pharmaceutical Bulletin*, *36*(4), 540–547. https://doi.org/10.1248/bpb.b12-00824

Aparicio, I. M., Garcia-Herreros, M., Fair, T., & Lonergan, P. (2010). Identification and regulation of glycogen synthase kinase-3 during bovine embryo development. *Reproduction (Cambridge, England)*, *140*(1), 83–92. https://doi.org/10.1530/REP-10-0040

Arias-Álvarez, M., García-García, R. M., Rebollar, P. G., Nicodemus, N., Revuelta, L., Millán, P., & Lorenzo, P. L. (2009). Effects of a lignin-rich fibre diet on productive, reproductive and endocrine parameters in nulliparous rabbit does. *Livestock Science*, *123*(2–3), 107–115. https://doi.org/10.1016/j.livsci.2008.10.013

Ashrafizadeh, M., Zarrabi, A., Saberifar, S., Hashemi, F., Hushmandi, K., Hashemi, F., Moghadam, E. R., Mohammadinejad, R., Najafi, M., & Garg, M. (2020). Nobiletin in Cancer Therapy: How This Plant Derived-Natural Compound Targets Various Oncogene and Onco-Suppressor Pathways. *Biomedicines*, *8*(5). https://doi.org/10.3390/biomedicines8050110

Ashry, M., Lee, K., Mondal, M., Datta, T. K., Folger, J. K., Rajput, S. K., Zhang, K., Hemeida, N. A., & Smith, G. W. (2015). Expression of TGFβ superfamily components and other markers of oocyte quality in oocytes selected by brilliant cresyl blue staining: Relevance to early embryonic development. *Molecular Reproduction and Development*, *82*(3), 251–264. https://doi.org/10.1002/mrd.22468

Ashry, M., Rajput, S. K., Folger, J. K., Knott, J. G., Hemeida, N. A., Kandil, O. M., Ragab, R. S., & Smith, G. W. (2018). Functional role of AKT signaling in bovine early embryonic development: Potential link to embryotrophic actions of follistatin. *Reproductive Biology and Endocrinology*, *16*(1), 1. https://doi.org/10.1186/s12958-017-0318-6

Ashry, M., Rajput, S. K., Folger, J. K., Yang, C., Knott, J. G., & Smith, G. W. (2020). Follistatin treatment modifies DNA methylation of the CDX2 gene in bovine preimplantation embryos. *Molecular Reproduction and Development*. https://doi.org/10.1002/mrd.23409

Assidi, M., Dieleman, S. J., & Sirard, M.-A. (2010). Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: Potential early markers of oocyte competence. *Reproduction (Cambridge, England)*, *140*(6), 835–852. https://doi.org/10.1530/REP-10-0248

Augustin, R., Pocar, P., Navarrete-Santos, A., Wrenzycki, C., Gandolfi, F., Niemann, H., & Fischer, B. (2001). Glucose transporter expression is developmentally regulated in in vitro derived bovine preimplantation embryos. *Molecular Reproduction and Development*, *60*(3), 370–376. https://doi.org/10.1002/mrd.1099

Averaimo, S., Milton, R. H., Duchen, M. R., & Mazzanti, M. (2010). Chloride intracellular channel 1 (CLIC1): Sensor and effector during oxidative stress. *FEBS Letters*, *584*(10), 2076–2084. https://doi.org/10.1016/j.febslet.2010.02.073

Badr, H., Bongioni, G., Abdoon, A. S. S., Kandil, O., & Puglisi, R. (2007). Gene expression in the in vitro-produced preimplantation bovine embryos. *Zygote (Cambridge, England)*, *15*(4), 355–367. https://doi.org/10.1017/S0967199407004315

Banwell, K. M., & Thompson, J. G. (2008). In vitro maturation of Mammalian oocytes: Outcomes and consequences. *Seminars in Reproductive Medicine*, *26*(2), 162–174. https://doi.org/10.1055/s-2008-1042955

Baran, V., Fabian, D., & Rehak, P. (2013). Akt/PKB plays role of apoptosis relay on entry into first mitosis of mouse embryo. *Zygote*, *21*(4), 406–416. https://doi.org/10.1017/S0967199413000178

Barja, G. (2007). Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: Implications for aging studies. *Rejuvenation Research*, *10*(2), 215–224. https://doi.org/10.1089/rej.2006.0516

Bavister, B. D. (2000). Interactions between embryos and the culture milieu. *Theriogenology*, *53*(2), 619–626. https://doi.org/10.1016/S0093-691X(99)00262-9

Belloc, E., Piqué, M., & Méndez, R. (2008). Sequential waves of polyadenylation and deadenylation define a translation circuit that drives meiotic progression. *Biochemical Society Transactions*, *36*(4), 665–670. https://doi.org/10.1042/BST0360665

Bennemann, J., Grothmann, H., & Wrenzycki, C. (2018). Reduced oxygen concentration during in vitro oocyte maturation alters global DNA methylation in the maternal pronucleus of subsequent zygotes in cattle. *Molecular Reproduction and Development*, *85*(11), 849–857. https://doi.org/10.1002/mrd.23073

Bermejo-Álvarez, P., Rizos, D., Rath, D., Lonergan, P., & Gutierrez-Adan, A. (2008). Epigenetic differences between male and female bovine blastocysts produced in vitro. *Physiological Genomics*, *32*(2), 264–272. https://doi.org/10.1152/physiolgenomics.00234.2007

Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P., & Gutierrez-Adan, A. (2010). Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proceedings of the National Academy of Sciences*, *107*(8), 3394–3399. https://doi.org/10.1073/pnas.0913843107

Bermejo-Alvarez, P., Roberts, R. M., & Rosenfeld, C. S. (2012). Effect of glucose concentration during in vitro culture of mouse embryos on development to blastocyst, success of embryo transfer, and litter sex ratio. *Molecular Reproduction and Development*, *79*(5), 329–336. https://doi.org/10.1002/mrd.22028

Bhutani, J., Sheikh, A., & Niazi, A. K. (2013). Akt inhibitors: Mechanism of action and implications for anticancer therapeutics. *Infectious Agents and Cancer*, *8*(1), 49. https://doi.org/10.1186/1750-9378-8-49

Bleil, J. D., & Wassarman, P. M. (1983). Sperm-egg interactions in the mouse: Sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Developmental Biology*, *95*(2), 317–324. https://doi.org/10.1016/0012-1606(83)90032-5

Blume-Jensen, P., & Hunter, T. (2001). Oncogenic kinase signalling. *Nature*, *411*(6835), 355–365. https://doi.org/10.1038/35077225

Boelhauve, M., Sinowatz, F., Wolf, E., & Paula-Lopes, F. F. (2005). Maturation of bovine oocytes in the presence of leptin improves development and reduces apoptosis of in vitro-produced blastocysts. *Biology of Reproduction*, *73*(4), 737–744. https://doi.org/10.1095/biolreprod.105.041103

Bondioli, K. R., Westhusin, M. E., & Looney, C. R. (1990). Production of identical bovine offspring by nuclear transfer. *Theriogenology*, *33*(1), 165–174. https://doi.org/10.1016/0093-691X(90)90607-U

Bouayed, J., & Bohn, T. (2010). Exogenous antioxidants--Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxidative Medicine and Cellular Longevity*, *3*(4), 228–237. https://doi.org/10.4161/oxim.3.4.12858

Boyacioglu, M., Turgut, H., Akgullu, C., Eryilmaz, U., Kum, C., & Onbasili, O. A. (2014). The effect of L-carnitine on oxidative stress responses of experimental contrast-induced nephropathy in rats. *The Journal of Veterinary Medical Science*, *76*(1), 1–8. https://doi.org/10.1292/jvms.13-0202

Brand, M. D., Orr, A. L., Perevoshchikova, I. V., & Quinlan, C. L. (2013). The role of mitochondrial function and cellular bioenergetics in ageing and disease. *British Journal of Dermatology*, *169*(s2), 1–8. https://doi.org/10.1111/bjd.12208

Brązert, M., Kranc, W., Nawrocki, M. J., Sujka-Kordowska, P., Konwerska, A., Jankowski, M., Kocherova, I., Celichowski, P., Jeseta, M., Ożegowska, K., Antosik, P., Bukowska, D., Skowroński, M. T., Bruska, M., Pawelczyk, L., Zabel, M., Piotrowska-Kempisty, H., Nowicki, M., & Kempisty, B. (2020). New markers for regulation of transcription and macromolecule metabolic process in porcine oocytes during in vitro maturation. *Molecular Medicine Reports*, *21*(3), 1537–1551. https://doi.org/10.3892/mmr.2020.10963

Brevini, T. a. L., Lonergan, P., Cillo, F., Francisci, C., Favetta, L. A., Fair, T., & Gandolfi, F. (2002). Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. *Molecular Reproduction and Development*, *63*(4), 510–517. https://doi.org/10.1002/mrd.10191

Bunel, A., Jorssen, E. P., Merckx, E., Leroy, J. L., Bols, P. E., & Sirard, M. A. (2015). Individual bovine in vitro embryo production and cumulus cell transcriptomic analysis to distinguish cumulus-oocyte complexes with high or low developmental potential. *Theriogenology*, *83*(2), 228–237. https://doi.org/10.1016/j.theriogenology.2014.09.019

Cagnone, G., & Sirard, M.-A. (2016). The embryonic stress response to in vitro culture: Insight from genomic analysis. *Reproduction*, *152*(6), R247–R261. https://doi.org/10.1530/REP-16-0391

Caixeta, E. S., Sutton-McDowall, M. L., Gilchrist, R. B., Thompson, J. G., Price, C. A., Machado, M. F., Lima, P. F., & Buratini, J. (2013). Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus–oocyte complexes. *Reproduction*, *146*(1), 27–35. https://doi.org/10.1530/REP-13-0079

Cajas, Y., Cañón-Beltrán, K., L.V. Leal, C., González, E., & Rizos, D. (2019). Nobiletin supplementation prior to EGA improves development and quality of bovine blastocysts in vitro. *Proceedings of the 35th Annual Meeting of the European Embryo Transfer Association (AETE)*, *v.16*, 686.

Cajas, Y. N., Cañón-Beltrán, K., Ladrón de Guevara, M., Millán de la Blanca, M. G., Ramos-Ibeas, P., Gutiérrez-Adán, A., Rizos, D., & González, E. M. (2020). Antioxidant Nobiletin Enhances Oocyte Maturation and Subsequent Embryo Development and Quality. *International Journal of Molecular Sciences*, *21*(15), 5340. https://doi.org/10.3390/ijms21155340

Camargo, L. S. A., Munk, M., Sales, J. N., Wohlres-Viana, S., Quintão, C. C. R., & Viana, J. H. M. (2019). Differential gene expression between in vivo and in vitro maturation: A comparative study with bovine oocytes derived from the same donor pool. *JBRA Assisted Reproduction*, *23*(1), 7–14. https://doi.org/10.5935/1518-0557.20180084

Cañón-Beltrán, K., Giraldo-Giraldo, J., Cajas, Y. N., Beltrán-Breña, P., Hidalgo, C. O., Vásquez, N., Leal, C. L. V., Gutiérrez-Adán, A., González, E. M., & Rizos, D. (2020). Inhibiting diacylglycerol acyltransferase-1 reduces lipid biosynthesis in bovine blastocysts produced in vitro. *Theriogenology*, *158*, 267–276. https://doi.org/10.1016/j.theriogenology.2020.09.014

Canovas, S., Ross, P. J., Kelsey, G., & Coy, P. (2017). DNA Methylation in Embryo Development: Epigenetic Impact of ART (Assisted Reproductive Technologies). *BioEssays*, *39*(11), 1700106. https://doi.org/10.1002/bies.201700106

Cantley, L. C. (2002). The Phosphoinositide 3-Kinase Pathway. *Science*, *296*(5573), 1655–1657. https://doi.org/10.1126/science.296.5573.1655

Cebrian-Serrano, A., Salvador, I., García-Roselló, E., Pericuesta, E., Pérez-Cerezales, S., Gutierrez-Adán, A., Coy, P., & Silvestre, M. A. (2013). Effect of the Bovine Oviductal Fluid on In Vitro Fertilization, Development and Gene Expression of In Vitro-Produced Bovine Blastocysts. *Reproduction in Domestic Animals*, *48*(2), 331–338. https://doi.org/10.1111/j.1439-0531.2012.02157.x

Cecconi, S., Mauro, A., Cellini, V., & Patacchiola, F. (2012). The role of Akt signalling in the mammalian ovary. *The International Journal of Developmental Biology*, *56*(10–12), 809–817. https://doi.org/10.1387/ijdb.120146sc

Cecconi, S., Rossi, G., Santilli, A., Stefano, L. D., Hoshino, Y., Sato, E., Palmerini, M. G., & Macchiarelli, G. (2010). Akt expression in mouse oocytes matured in vivo and in vitro. *Reproductive BioMedicine Online*, *20*(1), 35–41. https://doi.org/10.1016/j.rbmo.2009.10.011

Cetica, P. D., Pintos, L. N., Dalvit, G. C., & Beconi, M. T. (2001). Antioxidant enzyme activity and oxidative stress in bovine oocyte in vitro maturation. *IUBMB Life*, *51*(1), 57–64. https://doi.org/10.1080/15216540119253

Chen, C., Ono, M., Takeshima, M., & Nakano, S. (2014). Antiproliferative and apoptosis-inducing activity of nobiletin against three subtypes of human breast cancer cell lines. *Anticancer Research*, *34*(4), 1785–1792.

Chen, F., De Neubourg, D., Debrock, S., Peeraer, K., D'Hooghe, T., & Spiessens, C. (2016). Selecting the embryo with the highest implantation potential using a data mining based prediction model. *Reproductive Biology and Endocrinology : RB&E*, *14*. https://doi.org/10.1186/s12958-016-0145-1

Chen, Jianchu, Chen, A. Y., Huang, H., Ye, X., Rollyson, W. D., Perry, H. E., Brown, K. C., Rojanasakul, Y., Rankin, G. O., Dasgupta, P., & Chen, Y. C. (2015). The flavonoid nobiletin inhibits tumor growth and angiogenesis of ovarian cancers via the Akt pathway. *International Journal of Oncology*, *46*(6), 2629–2638. https://doi.org/10.3892/ijo.2015.2946

Chen, Junming, Lian, X., Du, J., Xu, S., Wei, J., Pang, L., Song, C., He, L., & Wang, S. (2016). Inhibition of phosphorylated Ser473-Akt from translocating into the nucleus contributes to 2-cell arrest and defective zygotic genome activation in mouse preimplantation embryogenesis. *Development, Growth & Differentiation*, *58*(3), 280–292. https://doi.org/10.1111/dgd.12273

Childs, S., Carter, F., Lynch, C. O., Sreenan, J. M., Lonergan, P., Hennessy, A. A., & Kenny, D. A. (2008). Embryo yield and quality following dietary supplementation of beef heifers with n-3 polyunsaturated fatty acids (PUFA). *Theriogenology*, *70*(6), 992–1003. https://doi.org/10.1016/j.theriogenology.2008.06.008

Cho, H. W., Jung, S. Y., Lee, G. H., Cho, J. H., & Choi, I. Y. (2015). Neuroprotective effect of Citrus unshiu immature peel and nobiletin inhibiting hydrogen peroxide-induced oxidative stress in HT22 murine hippocampal neuronal cells. *Pharmacognosy Magazine*, *11*(Suppl 2), S284–S289. https://doi.org/10.4103/0973-1296.166047

Choi, S.-Y., Hwang, J.-H., Ko, H.-C., Park, J.-G., & Kim, S.-J. (2007). Nobiletin from citrus fruit peel inhibits the DNA-binding activity of NF-κB and ROS production in LPS-activated RAW 264.7 cells. *Journal of Ethnopharmacology*, *113*(1), 149–155. https://doi.org/10.1016/j.jep.2007.05.021

Choi, Y., Kim, Y., Ham, H., Park, Y., Jeong, H.-S., & Lee, J. (2011). Nobiletin Suppresses Adipogenesis by Regulating the Expression of Adipogenic Transcription Factors and the Activation of AMP-Activated Protein Kinase (AMPK). *Journal of Agricultural and Food Chemistry*, *59*(24), 12843–12849. https://doi.org/10.1021/jf2033208

Chowdhury, M. M. R., Choi, B.-H., Khan, I., Lee, K.-L., Mesalam, A., Song, S.-H., Xu, L., Joo, M.-D., Afrin, F., & Kong, I.-K. (2017). Supplementation of lycopene in maturation media improves bovine embryo quality in vitro. *Theriogenology*, *103*, 173–184. https://doi.org/10.1016/j.theriogenology.2017.08.003

Chowdhury, M. M. R., Mesalam, A., Khan, I., Joo, M.-D., Lee, K.-L., Xu, L., Afrin, F., & Kong, I.-K. (2018). Improved developmental competence in embryos treated with lycopene during in vitro culture system. *Molecular Reproduction and Development*, *85*(1), 46–61. https://doi.org/10.1002/mrd.22937

Chronis, C., Fiziev, P., Papp, B., Butz, S., Bonora, G., Sabri, S., Ernst, J., & Plath, K. (2017). Cooperative Binding of Transcription Factors Orchestrates Reprogramming. *Cell*, *168*(3), 442-459.e20. https://doi.org/10.1016/j.cell.2016.12.016

Clemente, M., de La Fuente, J., Fair, T., Al Naib, A., Gutierrez-Adan, A., Roche, J. F., Rizos, D., & Lonergan, P. (2009). Progesterone and conceptus elongation in cattle: A direct effect on the embryo or an indirect effect via the endometrium? *REPRODUCTION*, *138*(3), 507–517. https://doi.org/10.1530/REP-09-0152

Collado, M. del, Saraiva, N. Z., Lopes, F. L., Gaspar, R. C., Padilha, L. C., Costa, R. R., Rossi, G. F., Vantini, R., & Garcia, J. M. (2015). Influence of bovine serum albumin and fetal bovine serum supplementation during in vitro maturation on lipid and mitochondrial behaviour in oocytes and lipid accumulation in bovine embryos. *Reproduction, Fertility and Development*, *28*(11), 1721–1732. https://doi.org/10.1071/RD15067

Combelles, C. M. H., Gupta, S., & Agarwal, A. (2009). Could oxidative stress influence the in-vitro maturation of oocytes? *Reproductive Biomedicine Online*, *18*(6), 864–880. https://doi.org/10.1016/s1472-6483(10)60038-7

Conti, M., Hsieh, M., Musa Zamah, A., & Oh, J. S. (2012). Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. *Molecular and Cellular Endocrinology*, *356*(1), 65–73. https://doi.org/10.1016/j.mce.2011.11.002

Corrêa, G. A., Rumpf, R., Mundim, T. C. D., Franco, M. M., & Dode, M. A. N. (2008). Oxygen tension during in vitro culture of bovine embryos: Effect in production and expression of genes related to oxidative stress. *Animal Reproduction Science*, *104*(2), 132–142. https://doi.org/10.1016/j.anireprosci.2007.02.002

Coticchio, G., Dal Canto, M., Mignini Renzini, M., Guglielmo, M. C., Brambillasca, F., Turchi, D., Novara, P. V., & Fadini, R. (2015). Oocyte maturation: Gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganization. *Human Reproduction Update*, *21*(4), 427–454. https://doi.org/10.1093/humupd/dmv011

Cummins, J. M. (2004). The role of mitochondria in the establishment of oocyte functional competence. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *115*, S23–S29. https://doi.org/10.1016/j.ejogrb.2004.01.011

Dabrundashvili, N. G., Kvaratskhelia, E. B., Gagua, M., Maisuradze, E., Chkhikvishvili, I., Zhuravliova, E. I., & Mikeladze, D. G. (2011). Nobiletin Transiently Increases the Production of Nitric Oxide and Changes the Activity of Succinate Dehydrogenase in Human Blood Lymphocytes. *Journal of Food Biochemistry*, *35*(2), 638–649. https://doi.org/10.1111/j.1745-4514.2010.00407.x

Dalvit, G., Llanes, S. P., Descalzo, A., Insani, M., Beconi, M., & Cetica, P. (2005). Effect of alpha-tocopherol and ascorbic acid on bovine oocyte in vitro maturation. *Reproduction in Domestic Animals = Zuchthygiene*, *40*(2), 93–97. https://doi.org/10.1111/j.1439-0531.2004.00522.x

de Matos, D. G., & Furnus, C. C. (2000). The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development: Effect of β-mercaptoethanol, cysteine and cystine. *Theriogenology*, *53*(3), 761–771. https://doi.org/10.1016/S0093-691X(99)00278-2

de Matos, D. G., Herrera, C., Cortvrindt, R., Smitz, J., Van Soom, A., Nogueira, D., & Pasqualini, R. S. (2002). Cysteamine supplementation during in vitro maturation and embryo culture: A useful tool for increasing the efficiency of bovine in vitro embryo production. *Molecular Reproduction and Development*, *62*(2), 203–209. https://doi.org/10.1002/mrd.10087

Degrelle, S. A., Campion, E., Cabau, C., Piumi, F., Reinaud, P., Richard, C., Renard, J.-P., & Hue, I. (2005). Molecular evidence for a critical period in mural trophoblast development in bovine blastocysts. *Developmental Biology*, *288*(2), 448–460. https://doi.org/10.1016/j.ydbio.2005.09.043

Deleuze, S., & Goudet, G. (2010). Cysteamine supplementation of in vitro maturation media: A review. *Reproduction in Domestic Animals = Zuchthygiene*, *45*(6), e476-482. https://doi.org/10.1111/j.1439-0531.2010.01587.x

dos Santos, E. C., Varchetta, R., de Lima, C. B., Ispada, J., Martinho, H. S., Fontes, P. K., Nogueira, M. F. G., Gasparrini, B., & Milazzotto, M. P. (2019). The effects of crocetin supplementation on the blastocyst outcome, transcriptomic and metabolic profile of in vitro produced bovine embryos. *Theriogenology*, *123*, 30–36. https://doi.org/10.1016/j.theriogenology.2018.08.010

Downs, S. M. (2015). Nutrient pathways regulating the nuclear maturation of mammalian oocytes. *Reproduction, Fertility and Development*, *27*(4), 572–582. https://doi.org/10.1071/RD14343

Downs, S. M., Humpherson, P. G., & Leese, H. J. (2002). Pyruvate utilization by mouse oocytes is influenced by meiotic status and the cumulus oophorus. *Molecular Reproduction and Development*, *62*(1), 113–123. https://doi.org/10.1002/mrd.10067

Driancourt, M. A. (2001). Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. *Theriogenology*, *55*(6), 1211–1239. https://doi.org/10.1016/S0093-691X(01)00479-4

Duan, J., Zhu, L., Dong, H., Zheng, X., Jiang, Z., Chen, J., & Tian, X. C. (2019). Analysis of mRNA abundance for histone variants, histone- and DNA-modifiers in bovine in vivo and in vitro oocytes and embryos. *Scientific Reports*, *9*(1), 1217. https://doi.org/10.1038/s41598-018-38083-4

Ducibella, T., & Buetow, J. (1994). Competence to Undergo Normal, Fertilization-Induced Cortical Activation Develops after Metaphase I of Meiosis in Mouse Oocytes. *Developmental Biology*, *165*(1), 95–104. https://doi.org/10.1006/dbio.1994.1237

Dummler, B., Tschopp, O., Hynx, D., Yang, Z.-Z., Dirnhofer, S., & Hemmings, B. A. (2006). Life with a single isoform of Akt: Mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. *Molecular and Cellular Biology*, *26*(21), 8042–8051. https://doi.org/10.1128/MCB.00722-06

Dunning, K. R., Cashman, K., Russell, D. L., Thompson, J. G., Norman, R. J., & Robker, R. L. (2010). Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biology of Reproduction*, *83*(6), 909–918. https://doi.org/10.1095/biolreprod.110.084145

Dunning, K. R., Russell, D. L., & Robker, R. L. (2014). Lipids and oocyte developmental competence: The role of fatty acids and β-oxidation. *Reproduction (Cambridge, England)*, *148*(1), R15-27. https://doi.org/10.1530/REP-13-0251

Ealy, A. D., & Yang, Q. E. (2009). REVIEW ARTICLE: Control of Interferon-Tau Expression During Early Pregnancy in Ruminants. *American Journal of Reproductive Immunology*, *61*(2), 95–106. https://doi.org/10.1111/j.1600-0897.2008.00673.x

Edson, M. A., Nagaraja, A. K., & Matzuk, M. M. (2009). The mammalian ovary from genesis to revelation. *Endocrine Reviews*, *30*(6), 624–712. https://doi.org/10.1210/er.2009-0012

Egerszegi, I., Alm, H., Rátky, J., Heleil, B., Brüssow, K.-P., & Torner, H. (2010). Meiotic progression, mitochondrial features and fertilisation characteristics of porcine oocytes with different G6PDH activities. *Reproduction, Fertility and Development*, *22*(5), 830–838. https://doi.org/10.1071/RD09140

Eichenlaub-Ritter, U., Wieczorek, M., Lüke, S., & Seidel, T. (2011). Age related changes in mitochondrial function and new approaches to study redox regulation in mammalian oocytes in response to age or maturation conditions. *Mitochondrion*, *11*(5), 783–796. https://doi.org/10.1016/j.mito.2010.08.011

El Hajj, N., & Haaf, T. (2013). Epigenetic disturbances in in vitro cultured gametes and embryos: Implications for human assisted reproduction. *Fertility and Sterility*, *99*(3), 632–641. https://doi.org/10.1016/j.fertnstert.2012.12.044

El-Raey, M., Geshi, M., Somfai, T., Kaneda, M., Hirako, M., Abdel-Ghaffar, A. E., Sosa, G. A., El-Roos, M. E. A. A., & Nagai, T. (2011). Evidence of melatonin synthesis in the cumulus oocyte complexes and its role in enhancing oocyte maturation in vitro in cattle. *Molecular Reproduction and Development*, *78*(4), 250–262. https://doi.org/10.1002/mrd.21295

Endo, M., Kawahara-Miki, R., Cao, F., Kimura, K., Kuwayama, T., Monji, Y., & Iwata, H. (2013). Estradiol supports in vitro development of bovine early antral follicles. *Reproduction*, *145*(1), 85–96. https://doi.org/10.1530/REP-12-0319

Eppig. (2001). Oocyte control of ovarian follicular development and function in mammals. *Reproduction*, *122*(6), 829–838. https://doi.org/10.1530/rep.0.1220829

Eppig, J. J., O'Brien, M. J., Wigglesworth, K., Nicholson, A., Zhang, W., & King, B. A. (2009). Effect of in vitro maturation of mouse oocytes on the health and lifespan of adult offspring. *Human Reproduction*, *24*(4), 922–928. https://doi.org/10.1093/humrep/den466

Eppig, John J. (1982). The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. *Developmental Biology*, *89*(1), 268–272. https://doi.org/10.1016/0012-1606(82)90314-1

Fair. (2003). Follicular oocyte growth and acquisition of developmental competence. *Animal Reproduction Science*, *78*(3), 203–216. https://doi.org/10.1016/S0378-4320(03)00091-5

Fair, Hyttel, P, & Greve, T. (1995). *Bovine oocyte diameter in relation to maturational competence and transcriptional activity. 42*((4),), 437-442. https://doi.org/10.1002/mrd.1080420410

Fair T. (2013, September). *Molecular and endocrine determinants of oocyte competence*. https://www.animal-reproduction.org/article/5b5a604af7783717068b469b

Fair, T., Lonergan, P., Dinnyes, A., Cottell, D. C., Hyttel, P., Ward, F. A., & Boland, M. P. (2001). Ultrastructure of bovine blastocysts following cryopreservation: Effect of method of blastocyst production. *Molecular Reproduction and Development*, *58*(2), 186–195. https://doi.org/10.1002/1098-2795(200102)58:2<186::AID-MRD8>3.0.CO;2-N

Fan, H.-Y., & Sun, Q.-Y. (2004). Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. *Biology of Reproduction*, *70*(3), 535–547. https://doi.org/10.1095/biolreprod.103.022830

Farin, P. W., Crosier, A. E., & Farin, C. E. (2001). Influence of in vitro systems on embryo survival and fetal development in cattle. *Theriogenology*, *55*(1), 151–170. https://doi.org/10.1016/S0093-691X(00)00452-0

Feng, S., Zhou, H., Wu, D., Zheng, D., Qu, B., Liu, R., Zhang, C., Li, Z., Xie, Y., & Luo, H.-B. (2020). Nobiletin and its derivatives overcome multidrug resistance (MDR) in cancer: Total synthesis and discovery of potent MDR reversal agents. *Acta Pharmaceutica Sinica. B*, *10*(2), 327–343. https://doi.org/10.1016/j.apsb.2019.07.007

Ferreira, C. R., Saraiva, S. A., Catharino, R. R., Garcia, J. S., Gozzo, F. C., Sanvido, G. B., Santos, L. F. A., Lo Turco, E. G., Pontes, J. H. F., Basso, A. C., Bertolla, R. P., Sartori, R., Guardieiro, M. M., Perecin, F., Meirelles, F. V., Sangalli, J. R., & Eberlin, M. N. (2010). Single embryo and oocyte lipid fingerprinting by mass spectrometry. *Journal of Lipid Research*, *51*(5), 1218–1227. https://doi.org/10.1194/jlr.D001768

Ferreira, E. M., Vireque, A. A., Adona, P. R., Meirelles, F. V., Ferriani, R. A., & Navarro, P. A. A. S. (2009). Cytoplasmic maturation of bovine oocytes: Structural and biochemical modifications and acquisition of developmental competence. *Theriogenology*, *71*(5), 836–848. https://doi.org/10.1016/j.theriogenology.2008.10.023

Filatov, M., Khramova, Y., & Semenova, M. (2019). Molecular Mechanisms of Prophase I Meiotic Arrest Maintenance and Meiotic Resumption in Mammalian Oocytes. *Reproductive Sciences (Thousand Oaks, Calif.)*, *26*(11), 1519–1537. https://doi.org/10.1177/1933719118765974

Fleming, T. P., Ghassemifar, M. R., & Sheth, B. (2000). Junctional Complexes in the Early Mammalian Embryo. *Seminars in Reproductive Medicine*, *18*(2), 185–194. https://doi.org/10.1055/s-2000-12557

Fleming, T. P., Kwong, W. Y., Porter, R., Ursell, E., Fesenko, I., Wilkins, A., Miller, D. J., Watkins, A. J., & Eckert, J. J. (2004). The Embryo and Its Future1. *Biology of Reproduction*, *71*(4), 1046–1054. https://doi.org/10.1095/biolreprod.104.030957

Forde, N., Carter, F., Fair, T., Crowe, M. A., Evans, A. C. O., Spencer, T. E., Bazer, F. W., McBride, R., Boland, M. P., O'Gaora, P., Lonergan, P., & Roche, J. F. (2009). Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. *Biology of Reproduction*, *81*(4), 784–794. https://doi.org/10.1095/biolreprod.108.074336

Gad, A., Schellander, K., Hoelker, M., & Tesfaye, D. (2012). Transcriptome profile of early mammalian embryos in response to culture environment. *Animal Reproduction Science*, *134*(1), 76–83. https://doi.org/10.1016/j.anireprosci.2012.08.014

Galli, C., Duchi, R., Crotti, G., Turini, P., Ponderato, N., Colleoni, S., Lagutina, I., & Lazzari, G. (2003). Bovine embryo technologies. *Theriogenology*, *59*(2), 599–616. https://doi.org/10.1016/S0093-691X(02)01243-8

Gao, A.-M., Ke, Z.-P., Shi, F., Sun, G.-C., & Chen, H. (2013). Chrysin enhances sensitivity of BEL-7402/ADM cells to doxorubicin by suppressing PI3K/Akt/Nrf2 and ERK/Nrf2 pathway. *Chemico-Biological Interactions*, *206*(1), 100–108. https://doi.org/10.1016/j.cbi.2013.08.008

Gardner, D. K., Wale, P. L., Collins, R., & Lane, M. (2011). Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Human Reproduction (Oxford, England)*, *26*(8), 1981–1986. https://doi.org/10.1093/humrep/der143

Gaspar, R. C., Arnold, D. R., Corrêa, C. A. P., da Rocha, C. V., Penteado, J. C. T., Del Collado, M., Vantini, R., Garcia, J. M., & Lopes, F. L. (2015). Oxygen tension affects histone remodeling of in vitro-produced embryos in a bovine model. *Theriogenology*, *83*(9), 1408–1415. https://doi.org/10.1016/j.theriogenology.2015.01.002

Ge, H., Tollner, T. L., Hu, Z., Dai, M., Li, X., Guan, H., Shan, D., Zhang, X., Lv, J., Huang, C., & Dong, Q. (2012). The importance of mitochondrial metabolic activity and mitochondrial DNA replication during oocyte maturation in vitro on oocyte quality and subsequent embryo developmental competence. *Molecular Reproduction and Development*, *79*(6), 392–401. https://doi.org/10.1002/mrd.22042

Geisert, R. D., & Bazer, F. W. (Eds.). (2015). The Role of Steroid Hormone Receptors in the Establishment of Pregnancy in Rodents. In *Regulation of Implantation and Establishment of Pregnancy in Mammals: Tribute to 45 Year Anniversary of Roger V. Short's "Maternal Recognition of Pregnancy"* (pp. 27–50). Springer International Publishing. https://doi.org/10.1007/978-3-319-15856-3_3

Ghanem, N., Ha, A.-N., Fakruzzaman, Md., Bang, J.-I., Lee, S.-C., & Kong, I.-K. (2014). Differential expression of selected candidate genes in bovine embryos produced in vitro and cultured with chemicals modulating lipid metabolism. *Theriogenology*, *82*(2), 238–250. https://doi.org/10.1016/j.theriogenology.2014.03.024

Goissis, M. D., & Cibelli, J. B. (2014). Functional characterization of CDX2 during bovine preimplantation development in vitro. *Molecular Reproduction and Development*, *81*(10), 962–970. https://doi.org/10.1002/mrd.22415

Goodchild, S. C., Howell, M. W., Cordina, N. M., Littler, D. R., Breit, S. N., Curmi, P. M. G., & Brown, L. J. (2009). Oxidation promotes insertion of the CLIC1 chloride intracellular channel into the membrane. *European Biophysics Journal*, *39*(1), 129. https://doi.org/10.1007/s00249-009-0450-0

Gordo, A. C., He, C. L., Smith, S., & Fissore, R. A. (2001). Mitogen activated protein kinase plays a significant role in metaphase II arrest, spindle morphology, and maintenance of maturation promoting factor activity in bovine oocytes. *Molecular Reproduction and Development*, *59*(1), 106–114. https://doi.org/10.1002/mrd.1012

Gordon, I. (2003). *Laboratory Production of Cattle Embryos*. CABI.

Graf, A., Krebs, S., Zakhartchenko, V., Schwalb, B., Blum, H., & Wolf, E. (2014). Fine mapping of genome activation in bovine embryos by RNA sequencing. *Proceedings of the National Academy of Sciences*, *111*(11), 4139–4144. https://doi.org/10.1073/pnas.1321569111

Guérin, P., El Mouatassim, S., & Ménézo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reproduction Update*, *7*(2), 175–189. https://doi.org/10.1093/humupd/7.2.175

Guillomot, M. (1995). Cellular interactions during implantation in domestic ruminants. *Journal of Reproduction and Fertility. Supplement*, *49*, 39–51.

Gülçin, İ. (2006). Antioxidant and antiradical activities of l-carnitine. *Life Sciences*, *78*(8), 803–811. https://doi.org/10.1016/j.lfs.2005.05.103

Gupta, S., Choi, A., Yu, H. Y., Czerniak, S. M., Holick, E. A., Paolella, L. J., Agarwal, A., & Combelles, C. M. H. (2011). Fluctuations in total antioxidant capacity, catalase activity and hydrogen peroxide levels of follicular fluid during bovine folliculogenesis. *Reproduction, Fertility, and Development*, *23*(5), 673–680. https://doi.org/10.1071/RD10270

Hackett, A. J., Durnford, R., Mapletoft, R. J., & Marcus, G. J. (1993). Location and status of embryos in the genital tract of superovulated cows 4 to 6 days after insemination. *Theriogenology*, *40*(6), 1147–1153. https://doi.org/10.1016/0093-691X(93)90285-D

Hamdi, M., Lopera-Vasquez, R., Maillo, V., Sanchez-Calabuig, M. J., Núnez, C., Gutierrez-Adan, A., & Rizos, D. (2018). Bovine oviductal and uterine fluid support in vitro embryo development. *Reproduction, Fertility and Development*, *30*(7), 935–945. https://doi.org/10.1071/RD17286

Hamdi, M., Sánchez-Calabuig, M. J., Rodríguez-Alonso, B., Arnal, S. B., Roussi, K., Sturmey, R., Gutiérrez-Adán, A., Lonergan, P., & Rizos, D. (2019). Gene expression and metabolic response of bovine oviduct epithelial cells to the early embryo. *Reproduction*, *158*(1), 85–94. https://doi.org/10.1530/REP-18-0561

Harris, C., & Hansen, J. M. (2012). Oxidative Stress, Thiols, and Redox Profiles. In C. Harris & J. M. Hansen (Eds.), *Developmental Toxicology: Methods and Protocols* (pp. 325–346). Humana Press. https://doi.org/10.1007/978-1-61779-867-2_21

Harvey, A. J. (2007). The role of oxygen in ruminant preimplantation embryo development and metabolism. *Animal Reproduction Science*, *98*(1–2), 113–128. https://doi.org/10.1016/j.anireprosci.2006.10.008

Harvey, Alexandra J. (2019). Mitochondria in early development: Linking the microenvironment, metabolism and the epigenome. *Reproduction*, *157*(5), R159–R179. https://doi.org/10.1530/REP-18-0431

Hashimoto, S. (2009). Application of In Vitro Maturation to Assisted Reproductive Technology. *Journal of Reproduction and Development*, *55*(1), 1–10. https://doi.org/10.1262/jrd.20127

Hashimoto, S., Minami, N., Yamada, M., & Imai, H. (2000). Excessive concentration of glucose during in vitro maturation impairs the developmental competence of bovine oocytes after in vitro fertilization: Relevance to intracellular reactive oxygen species and glutathione contents. *Molecular Reproduction and Development*, *56*(4), 520–526. https://doi.org/10.1002/1098-2795(200008)56:4<520::AID-MRD10>3.0.CO;2-0

He, L., He, T., Farrar, S., Ji, L., Liu, T., & Ma, X. (2017). Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species. *Cellular Physiology and Biochemistry*, *44*(2), 532–553. https://doi.org/10.1159/000485089

He, Z., Li, B., Rankin, G. O., Rojanasakul, Y., & Chen, Y. C. (2015). Selecting bioactive phenolic compounds as potential agents to inhibit proliferation and VEGF expression in human ovarian cancer cells. *Oncology Letters*, *9*(3), 1444–1450. https://doi.org/10.3892/ol.2014.2818

Heinzmann, J., Hansmann, T., Herrmann, D., Wrenzycki, C., Zechner, U., Haaf, T., & Niemann, H. (2011). Epigenetic profile of developmentally important genes in bovine oocytes. *Molecular Reproduction and Development*, *78*(3), 188–201. https://doi.org/10.1002/mrd.21281

Holm, P., Booth, P. J., Schmidt, M. H., Greve, T., & Callesen, H. (1999). High bovine blastocyst development in a static in vitro production system using sofaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology*, *52*(4), 683–700. https://doi.org/10.1016/S0093-691X(99)00162-4

Hoodbhoy, T., Dandekar, P., Calarco, P., & Talbot, P. (2001). P62/p56 are cortical granule proteins that contribute to formation of the cortical granule envelope and play a role in mammalian preimplantation development. *Molecular Reproduction and Development*, *59*(1), 78–89. https://doi.org/10.1002/mrd.1009

Horigome, S., Maeda, M., Ho, H.-J., Shirakawa, H., & Komai, M. (2016). Effect of Kaempferia parviflora extract and its polymethoxyflavonoid components on testosterone production in mouse testis-derived tumour cells. *Journal of Functional Foods*, *26*, 529–538. https://doi.org/10.1016/j.jff.2016.08.008

Hoshino, Y., & Sato, E. (2008). Protein kinase B (PKB/Akt) is required for the completion of meiosis in mouse oocytes. *Developmental Biology*, *314*(1), 215–223. https://doi.org/10.1016/j.ydbio.2007.12.005

Hosoe, M., & shioya, Y. (1997). Distribution of cortical granules in bovine oocytes classified by cumulus complex. *Zygote*, *5*(4), 371–376. https://doi.org/10.1017/S0967199400003956

Hsieh, M., Zamah, A. M., & Conti, M. (2009). Epidermal Growth Factor-Like Growth Factors in the Follicular Fluid: Role in Oocyte Development and Maturation. *Seminars in Reproductive Medicine*, *27*(1), 52–61. https://doi.org/10.1055/s-0028-1108010

Hu, J., Cheng, D., Gao, X., Bao, J., Ma, X., & Wang, H. (2012). Vitamin C Enhances the In vitro Development of Porcine Pre-implantation Embryos by Reducing Oxidative Stress. *Reproduction in Domestic Animals*, *47*(6), 873–879. https://doi.org/10.1111/j.1439-0531.2011.01982.x

Huang, H., Li, L., Shi, W., Liu, H., Yang, J., Yuan, X., & Wu, L. (2016a). The multifunctional effects of nobiletin and its metabolites in vivo and in vitro. *Evidence-Based Complementary and Alternative Medicine*, *2016*, 1–14. https://doi.org/10.1155/2016/2918796

Huang, H., Li, L., Shi, W., Liu, H., Yang, J., Yuan, X., & Wu, L. (2016b, September 27). *The Multifunctional Effects of Nobiletin and Its Metabolites In Vivo and In Vitro* [Review Article]. Evidence-Based Complementary and Alternative Medicine; Hindawi. https://doi.org/10.1155/2016/2918796

Hue, I., Degrelle, S. A., & Turenne, N. (2012). Conceptus elongation in cattle: Genes, models and questions. *Animal Reproduction Science*, *134*(1), 19–28. https://doi.org/10.1016/j.anireprosci.2012.08.007

Hussein, T. S., Sutton-McDowall, M. L., Gilchrist, R. B., & Thompson, J. G. (2011). Temporal effects of exogenous oocyte-secreted factors on bovine oocyte developmental competence during IVM. *Reproduction, Fertility and Development*, *23*(4), 576–584. https://doi.org/10.1071/RD10323

Hutchinson, J. A., Shanware, N. P., Chang, H., & Tibbetts, R. S. (2011). Regulation of Ribosomal Protein S6 Phosphorylation by Casein Kinase 1 and Protein Phosphatase 1. *The Journal of Biological Chemistry*, *286*(10), 8688–8696. https://doi.org/10.1074/jbc.M110.141754

Ihara, H., Yamamoto, H., Ida, T., Tsutsuki, H., Sakamoto, T., Fujita, T., Okada, T., & Kozaki, S. (2012). Inhibition of Nitric Oxide Production and Inducible Nitric Oxide Synthase Expression by a Polymethoxyflavone from Young Fruits of *Citrus unshiu* in Rat Primary Astrocytes. *Bioscience, Biotechnology, and Biochemistry*, *advpub*. https://doi.org/10.1271/bbb.120215

Inoue, M., Naito, K., Nakayama, T., & Sato, E. (1998). Mitogen-activated protein kinase translocates into the germinal vesicle and induces germinal vesicle breakdown in porcine oocytes. *Biology of Reproduction*, *58*(1), 130–136. https://doi.org/10.1095/biolreprod58.1.130

Ireland, J. J. (1987). Control of follicular growth and development. *Journal of Reproduction and Fertility. Supplement*, *34*, 39–54.

Jang, C.-W., Shibata, Y., Starmer, J., Yee, D., & Magnuson, T. (2015). Histone H3.3 maintains genome integrity during mammalian development. *Genes & Development*, *29*(13), 1377–1392. https://doi.org/10.1101/gad.264150.115

Javed, M. H., & Wright, R. W. (1991). Determination of pentose phosphate and Embden-Meyerhof pathway activities in bovine embryos. *Theriogenology*, *35*(5), 1029–1037. https://doi.org/10.1016/0093-691x(91)90312-2

Jeong, C.-H., & Joo, S. H. (2016). Downregulation of Reactive Oxygen Species in Apoptosis. *Journal of Cancer Prevention*, *21*(1), 13–20. https://doi.org/10.15430/JCP.2016.21.1.13

Jeong, W., Jung, S., Bazer, F. W., & Kim, J. (2017). Stem cell factor-induced AKT cell signaling pathway: Effects on porcine trophectoderm and uterine luminal epithelial cells. *General and Comparative Endocrinology*, *250*, 113–121. https://doi.org/10.1016/j.ygcen.2017.05.015

Johnson, M. H., & Ziomek, C. A. (1981). The foundation of two distinct cell lineages within the mouse morula. *Cell*, *24*(1), 71–80. https://doi.org/10.1016/0092-8674(81)90502-x

Jojua, N., Sharikadze, N., Zhuravliova, E., Zaalishvili, E., & Mikeladze, D. G. (2015). Nobiletin restores impaired hippocampal mitochondrial bioenergetics in hypothyroidism through activation of matrix substrate-level phosphorylation. *Nutritional Neuroscience*, *18*(5), 225–231. https://doi.org/10.1179/1476830514Y.0000000120

Kalinowski, R. R., Berlot, C. H., Jones, T. L. Z., Ross, L. F., Jaffe, L. A., & Mehlmann, L. M. (2004). Maintenance of meiotic prophase arrest in vertebrate oocytes by a Gs protein-mediated pathway. *Developmental Biology*, *267*(1), 1–13. https://doi.org/10.1016/j.ydbio.2003.11.011

Kalous, J., Kubelka, M., Šolc, P., Šušor, A., & Motlík, J. (2009). AKT (protein kinase B) is implicated in meiotic maturation of porcine oocytes. *REPRODUCTION*, *138*(4), 645–654. https://doi.org/10.1530/REP-08-0461

Kang, J.-T., Kwon, D.-K., Park, S.-J., Kim, S.-J., Moon, J.-H., Koo, O.-J., Jang, G., & Lee, B.-C. (2013). Quercetin improves the in vitro development of porcine oocytes by decreasing reactive oxygen species levels. *Journal of Veterinary Science*, *14*(1), 15–20. https://doi.org/10.4142/jvs.2013.14.1.15

Kang, J.-T., Moon, J. H., Choi, J.-Y., Park, S. J., Kim, S. J., Saadeldin, I. M., & Lee, B. C. (2016). Effect of antioxidant flavonoids (Quercetin and Taxifolin) on in vitro maturation of porcine oocytes. *Asian-Australasian Journal of Animal Sciences*, *29*(3), 352–358. https://doi.org/10.5713/ajas.15.0341

Kathirvel, M., Soundian, E., & Kumanan, V. (2013). Differential expression dynamics of Growth differentiation factor9 (GDF9) and Bone morphogenetic factor15 (BMP15) mRNA transcripts during in vitro maturation of buffalo (Bubalus bubalis) cumulus–oocyte complexes. *SpringerPlus*, *2*(1), 206. https://doi.org/10.1186/2193-1801-2-206

Kawanishi, S., Oikawa, S., & Murata, M. (2005). Evaluation for safety of antioxidant chemopreventive agents. *Antioxidants & Redox Signaling*, *7*(11–12), 1728–1739. https://doi.org/10.1089/ars.2005.7.1728

Kawashima, A., Sekizawa, A., Koide, K., Hasegawa, J., Satoh, K., Arakaki, T., Takenaka, S., & Matsuoka, R. (2015). Vitamin C Induces the Reduction of Oxidative Stress and Paradoxically Stimulates the Apoptotic Gene Expression in Extravillous Trophoblasts Derived From First-Trimester Tissue. *Reproductive Sciences (Thousand Oaks, Calif.)*, *22*(7), 783–790. https://doi.org/10.1177/1933719114561561

Kent, L. N., Rumi, M. A. K., Kubota, K., Lee, D.-S., & Soares, M. J. (2011). FOSL1 Is Integral to Establishing the Maternal-Fetal Interface. *Molecular and Cellular Biology*, *31*(23), 4801–4813. https://doi.org/10.1128/MCB.05780-11

Kere, M., Siriboon, C., Lo, N.-W., Nguyen, N. T., & Ju, J.-C. (2012a). Ascorbic Acid Improves the Developmental Competence of Porcine Oocytes after Parthenogenetic Activation and Somatic Cell Nuclear Transplantation. *Journal of Reproduction and Development*, *advpub*. https://doi.org/10.1262/jrd.2012-114

Kere, M., Siriboon, C., Lo, N.-W., Nguyen, N. T., & Ju, J.-C. (2012b). Ascorbic acid improves the developmental competence of porcine oocytes after parthenogenetic activation and somatic cell nuclear transplantation. *Journal of Reproduction and Development*, *advpub*. https://doi.org/10.1262/jrd.2012-114

Khatib, H., & Gross, N. (2019). Symposium review: Embryo survival—A genomic perspective of the other side of fertility*. *Journal of Dairy Science*, *102*(4), 3744–3753. https://doi.org/10.3168/jds.2018-15252

Khurana, N. K., & Niemann, H. (2000). Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. *Biology of Reproduction*, *62*(4), 847–856. https://doi.org/10.1095/biolreprod62.4.847

Kimura, J., Nemoto, K., Degawa, M., Yokosuka, A., Mimaki, Y., Shimizu, K., Oku, N., & Ohizumi, Y. (2014). Upregulation of N-Methyl-D-aspartate Receptor Subunits and c-Fos Expressing Genes in PC12D Cells by Nobiletin. *Biological and Pharmaceutical Bulletin*, *37*(9), 1555–1558. https://doi.org/10.1248/bpb.b14-00177

Kimura, O., Ohta, C., Koga, N., Haraguchi, K., Kato, Y., & Endo, T. (2014a). Carrier-mediated uptake of nobiletin, a citrus polymethoxyflavonoid, in human intestinal Caco-2 cells. *Food Chemistry*, *154*, 145–150. https://doi.org/10.1016/j.foodchem.2013.12.069

Kimura, O., Ohta, C., Koga, N., Haraguchi, K., Kato, Y., & Endo, T. (2014b). Carrier-mediated uptake of nobiletin, a citrus polymethoxyflavonoid, in human intestinal Caco-2 cells. *Food Chemistry*, *154*, 145–150. https://doi.org/10.1016/j.foodchem.2013.12.069

Kimura, T., Tomooka, M., Yamano, N., Murayama, K., Matoba, S., Umehara, H., Kanai, Y., & Nakano, T. (2008). AKT signaling promotes derivation of embryonic germ cells from primordial germ cells. *Development (Cambridge, England)*, *135*(5), 869–879. https://doi.org/10.1242/dev.013474

Kovacic, P., & Somanathan, R. (2010). Multifaceted approach to resveratrol bioactivity: Focus on antioxidant action, cell signaling and safety. *Oxidative Medicine and Cellular Longevity*, *3*(2), 86–100. https://doi.org/10.4161/oxim.3.2.11147

Krischek, C., & Meinecke, B. (2002). In vitro maturation of bovine oocytes requires polyadenylation of mRNAs coding proteins for chromatin condensation, spindle assembly, MPF and MAP kinase activation. *Animal Reproduction Science*, *73*(3), 129–140. https://doi.org/10.1016/S0378-4320(02)00131-8

Krisher, R. L., & Bavister, B. D. (1998). Responses of oocytes and embryos to the culture environment. *Theriogenology*, *49*(1), 103–114. https://doi.org/10.1016/S0093-691X(97)00405-6

Kues, W. A., Sudheer, S., Herrmann, D., Carnwath, J. W., Havlicek, V., Besenfelder, U., Lehrach, H., Adjaye, J., & Niemann, H. (2008). Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(50), 19768–19773. https://doi.org/10.1073/pnas.0805616105

Kuijk, E. W., Tol, L. T. A. van, Velde, H. V. de, Wubbolts, R., Welling, M., Geijsen, N., & Roelen, B. A. J. (2012). The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. *Development*, *139*(5), 871–882. https://doi.org/10.1242/dev.071688

Kunimasa, K., Ikekita, M., Sato, M., Ohta, T., Yamori, Y., Ikeda, M., & Oikawa, T. (2010). *Nobiletin, a citrus polymethoxyflavonoid, suppresses multiple angiogenesis-related endothelial cell functions and angiogenesis in vivo—Kunimasa—2010—Cancer Science—Wiley Online Library*. https://onlinelibrary.wiley.com/doi/full/10.1111/j.1349-7006.2010.01668.x

Kwak, S.-S., Cheong, S.-A., Jeon, Y., Lee, E., Choi, K.-C., Jeung, E.-B., & Hyun, S.-H. (2012). The effects of resveratrol on porcine oocyte in vitro maturation and subsequent embryonic development after parthenogenetic activation and in vitro fertilization. *Theriogenology*, *78*(1), 86–101. https://doi.org/10.1016/j.theriogenology.2012.01.024

Lam, K. H., Alex, D., Lam, I. K., Tsui, S. K. W., Yang, Z. F., & Lee, S. M. Y. (2011). Nobiletin, a polymethoxylated flavonoid from citrus, shows anti-angiogenic activity in a zebrafish in vivo model and HUVEC in vitro model. *Journal of Cellular Biochemistry*, *112*(11), 3313–3321. https://doi.org/10.1002/jcb.23257

Landau, S., Braw-Tal, R., Kaim, M., Bor, A., & Bruckental, I. (2000). Preovulatory follicular status and diet affect the insulin and glucose content of follicles in high-yielding dairy cows. *Animal Reproduction Science*, *64*(3–4), 181–197. https://doi.org/10.1016/s0378-4320(00)00212-8

Laurincik, J., Kopecny, V., & Hyttel, P. (1994). Pronucleus development and DNA synthesis in bovine zygotes in vivo. *Theriogenology*, *42*(8), 1285–1293. https://doi.org/10.1016/0093-691X(94)90248-H

Lazzari, G., Wrenzycki, C., Herrmann, D., Duchi, R., Kruip, T., Niemann, H., & Galli, C. (2002). Cellular and Molecular Deviations in Bovine In Vitro-Produced Embryos Are Related to the Large Offspring Syndrome. *Biology of Reproduction*, *67*(3), 767–775. https://doi.org/10.1095/biolreprod.102.004481

Lee, B.-K., Uprety, N., Jang, Y. J., Tucker, S. K., Rhee, C., LeBlanc, L., Beck, S., & Kim, J. (2018). Fosl1 overexpression directly activates trophoblast-specific gene expression programs in embryonic stem cells. *Stem Cell Research*, *26*, 95–102. https://doi.org/10.1016/j.scr.2017.12.004

Lee, M. T., Bonneau, A. R., Takacs, C. M., Bazzini, A. A., DiVito, K. R., Fleming, E. S., & Giraldez, A. J. (2013). Nanog, Pou5f1 and SoxB1 activate zygotic gene expression during the maternal-to-zygotic transition. *Nature*, *503*(7476), 360–364. https://doi.org/10.1038/nature12632

Lee, S. H., Oh, H. J., Kim, M. J., Kim, G. A., Choi, Y. B., Jo, Y. K., Setyawan, E. M. N., & Lee, B. C. (2017). Oocyte maturation-related gene expression in the canine oviduct, cumulus cells, and oocytes and effect of co-culture with oviduct cells on in vitro maturation of oocytes. *Journal of Assisted Reproduction and Genetics*, *34*(7), 929–938. https://doi.org/10.1007/s10815-017-0910-x

Lee, Y.-S., Cha, B.-Y., Choi, S.-S., Choi, B.-K., Yonezawa, T., Teruya, T., Nagai, K., & Woo, J.-T. (2013). Nobiletin improves obesity and insulin resistance in high-fat diet-induced obese mice. *The Journal of Nutritional Biochemistry*, *24*(1), 156–162. https://doi.org/10.1016/j.jnutbio.2012.03.014

Leese, H. J. (2003). What does an embryo need? *Human Fertility (Cambridge, England)*, *6*(4), 180–185. https://doi.org/10.1080/1464770312331369463

Lequarre, A. S., Marchandise, J., Moreau, B., Massip, A., & Donnay, I. (2003). Cell Cycle Duration at the Time of Maternal Zygotic Transition for In Vitro Produced Bovine Embryos: Effect of Oxygen Tension and Transcription Inhibition. *Biology of Reproduction*, *69*(5), 1707–1713. https://doi.org/10.1095/biolreprod.103.017178

Leroy, J. L. M. R., Rizos, D., Sturmey, R., Bossaert, P., Gutierrez-Adan, A., Hoeck, V. V., Valckx, S., & Bols, P. E. J. (2011). Intrafollicular conditions as a major link between maternal metabolism and oocyte quality: A focus on dairy cow fertility. *Reproduction, Fertility and Development*, *24*(1), 1–12. https://doi.org/10.1071/RD11901

Li, S., Wang, H., Guo, L., Zhao, H., & Ho, C.-T. (2014). Chemistry and bioactivity of nobiletin and its metabolites. *Journal of Functional Foods*, *6*, 2–10. https://doi.org/10.1016/j.jff.2013.12.011

Li, S.-H., Lin, M.-H., Hwu, Y.-M., Lu, C.-H., Yeh, L.-Y., Chen, Y.-J., & Lee, R. K.-K. (2015). Correlation of cumulus gene expression of GJA1, PRSS35, PTX3, and SERPINE2 with oocyte maturation, fertilization, and embryo development. *Reproductive Biology and Endocrinology*, *13*(1), 93. https://doi.org/10.1186/s12958-015-0091-3

Lien, L.-M., Wang, M.-J., Chen, R.-J., Chiu, H.-C., Wu, J.-L., Shen, M.-Y., Chou, D.-S., Sheu, J.-R., Lin, K.-H., & Lu, W.-J. (2016). Nobiletin, a Polymethoxylated Flavone, Inhibits Glioma Cell Growth and Migration via Arresting Cell Cycle and Suppressing MAPK and Akt Pathways. *Phytotherapy Research*, *30*(2), 214–221. https://doi.org/10.1002/ptr.5517

Lim, W., & Song, G. (2016). Naringenin-induced migration of embrynoic trophectoderm cells is mediated via PI3K/AKT and ERK1/2 MAPK signaling cascades. *Molecular and Cellular Endocrinology*, *428*, 28–37. https://doi.org/10.1016/j.mce.2016.03.018

Lima, F. E. O., Bezerra, F. T. G., Souza, G. B., Matos, M. H. T., Hurk, R. van den, & Silva, J. R. V. (2018). Influence of interleukin 1 beta and tumour necrosis factor alpha on the in vitro growth, maturation and mitochondrial distribution of bovine oocytes from small antral follicles. *Zygote*, *26*(5), 381–387. https://doi.org/10.1017/S0967199418000382

Lin, Y., Mahan, K., Lathrop, W. F., Myles, D. G., & Primakoff, P. (1994). A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *Journal of Cell Biology*, *125*(5), 1157–1163. https://doi.org/10.1083/jcb.125.5.1157

Lin, Yuguang, Vermeer, M. A., Bos, W., van Buren, L., Schuurbiers, E., Miret-Catalan, S., & Trautwein, E. A. (2011). Molecular Structures of Citrus Flavonoids Determine Their Effects on Lipid Metabolism in HepG2 Cells by Primarily Suppressing ApoB Secretion. *Journal of Agricultural and Food Chemistry*, *59*(9), 4496–4503. https://doi.org/10.1021/jf1044475

Lindeman, L. C., Andersen, I. S., Reiner, A. H., Li, N., Aanes, H., Østrup, O., Winata, C., Mathavan, S., Müller, F., Aleström, P., & Collas, P. (2011). Prepatterning of Developmental Gene Expression by Modified Histones before Zygotic Genome Activation. *Developmental Cell*, *21*(6), 993–1004. https://doi.org/10.1016/j.devcel.2011.10.008

Liu, L., Rajareddy, S., Reddy, P., Jagarlamudi, K., Du, C., Shen, Y., Guo, Y., Boman, K., Lundin, E., Ottander, U., Selstam, G., & Liu, K. (2007). Phosphorylation and inactivation of glycogen synthase kinase-3 by soluble kit ligand in mouse oocytes during early follicular development. *Journal of Molecular Endocrinology*, *38*(1), 137–146. https://doi.org/10.1677/jme.1.02027

Liu, P., Begley, M., Michowski, W., Inuzuka, H., Ginzberg, M., Gao, D., Tsou, P., Gan, W., Papa, A., Kim, B. M., Wan, L., Singh, A., Zhai, B., Yuan, M., Wang, Z., Gygi, S. P., Lee, T. H., Lu, K.-P., Toker, A., … Wei, W. (2014). Cell-cycle-regulated activation of Akt kinase by phosphorylation at its carboxyl terminus. *Nature*, *508*(7497), 541–545. https://doi.org/10.1038/nature13079

Lonergan, Khatir H, Piumi F, Rieger D, Humblot P, & Boland MP. (1999, 117( ):159-167). *Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos—PubMed*. J Reprod Fertil. https://pubmed.ncbi.nlm.nih.gov/10645257/

Lonergan, P. (2007). State-of-the-art embryo technologies in cattle. *Society of Reproduction and Fertility Supplement*, *64*, 315–325. https://doi.org/10.5661/rdr-vi-315

Lonergan, P., & Fair, T. (2008). In vitro-produced bovine embryos—Dealing with the warts. *Theriogenology*, *69*(1), 17–22. https://doi.org/10.1016/j.theriogenology.2007.09.007

Lonergan, P., Monaghan, P., Rizos, D., Boland, M. P., & Gordon, I. (1994). Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in

vitro. *Molecular Reproduction and Development*, *37*(1), 48–53. https://doi.org/10.1002/mrd.1080370107

Lonergan, P., Rizos, D., Gutiérrez-Adán, A., Moreira, P. M., Pintado, B., de la Fuente, J., & Boland, M. P. (2003). Temporal Divergence in the Pattern of Messenger RNA Expression in Bovine Embryos Cultured from the Zygote to Blastocyst Stage In Vitro or In Vivo. *Biology of Reproduction*, *69*(4), 1424–1431. https://doi.org/10.1095/biolreprod.103.018168

Lonergan, Pat, Fair, T., Forde, N., & Rizos, D. (2016). Embryo development in dairy cattle. *Theriogenology*, *86*(1), 270–277. https://doi.org/10.1016/j.theriogenology.2016.04.040

Lonergan, Patrick, & Fair, T. (2016). Maturation of Oocytes in Vitro. *Annual Review of Animal Biosciences*, *4*(1), 255–268. https://doi.org/10.1146/annurev-animal-022114-110822

Loos, F. de, Vliet, C. van, Maurik, P. van, & Kruip, T. A. M. (1989). Morphology of immature bovine oocytes. *Gamete Research*, *24*(2), 197–204. https://doi.org/10.1002/mrd.1120240207

Lopera-Vásquez, R., Hamdi, M., Fernandez-Fuertes, B., Maillo, V., Beltrán-Breña, P., Calle, A., Redruello, A., López-Martín, S., Gutierrez-Adán, A., Yañez-Mó, M., Ramirez, M. Á., & Rizos, D. (2016). Extracellular Vesicles from BOEC in In Vitro Embryo Development and Quality. *PloS One*, *11*(2), e0148083. https://doi.org/10.1371/journal.pone.0148083

Lopera-Vasquez, R., Hamdi, M., Maillo, V., Lloreda, V., Coy, P., Gutierrez-Adan, A., Bermejo-Alvarez, P., & Rizos, D. (2017). Effect of bovine oviductal fluid on development and quality of bovine embryos produced in vitro. *Reproduction, Fertility and Development*, *29*(3), 621–629. https://doi.org/10.1071/RD15238

Lopes, A. S., Madsen, S. E., Ramsing, N. B., Løvendahl, P., Greve, T., & Callesen, H. (2007). Investigation of respiration of individual bovine embryos produced in vivo and in vitro and correlation with viability following transfer. *Human Reproduction (Oxford, England)*, *22*(2), 558–566. https://doi.org/10.1093/humrep/del404

López-Cardona, Sánchez-Calabuig, M. J., Beltran-Breña, P., Agirregoitia, N., Rizos, D., Agirregoitia, E., & Gutierrez-Adán, A. (2016). Exocannabinoids effect on in vitro bovine oocyte maturation via activation of AKT and ERK1/2. *Reproduction*, *152*(6), 603–612. https://doi.org/10.1530/REP-16-0199

Machaty, Z., Peippo, J., & Peter, A. (2012). Production and manipulation of bovine embryos: Techniques and terminology. *Theriogenology*, *78*(5), 937–950. https://doi.org/10.1016/j.theriogenology.2012.04.003

Madan, P., Calder, M. D., & Watson, A. J. (2005). Mitogen-activated protein kinase (MAPK) blockade of bovine preimplantation embryogenesis requires inhibition of both p38 and extracellular signal-regulated kinase (ERK) pathways. *Reproduction (Cambridge, England)*, *130*(1), 41–51. https://doi.org/10.1530/rep.1.00554

Magnuson, B., Ekim, B., & Fingar, D. C. (2011). Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochemical Journal*, *441*(1), 1–21. https://doi.org/10.1042/BJ20110892

Manning, B. D., & Cantley, L. C. (2007). AKT/PKB Signaling: Navigating Downstream. *Cell*, *129*(7), 1261–1274. https://doi.org/10.1016/j.cell.2007.06.009

Manning, B. D., & Toker, A. (2017). AKT/PKB Signaling: Navigating the Network. *Cell*, *169*(3), 381–405. https://doi.org/10.1016/j.cell.2017.04.001

Manthey, J. A., & Grohmann, K. (2001). Phenols in Citrus Peel Byproducts. Concentrations of Hydroxycinnamates and Polymethoxylated Flavones in Citrus Peel Molasses. *Journal of Agricultural and Food Chemistry*, *49*(7), 3268–3273. https://doi.org/10.1021/jf010011r

Marques, L., & Thorsteinsdóttir, S. (2013). Dynamics of Akt activation during mouse embryo development: Distinct subcellular patterns distinguish proliferating versus differentiating cells. *Differentiation; Research in Biological Diversity*, *86*(1–2), 48–56. https://doi.org/10.1016/j.diff.2013.07.001

Mehlmann, L. M. (2005). Stops and starts in mammalian oocytes: Recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction (Cambridge, England)*, *130*(6), 791–799. https://doi.org/10.1530/rep.1.00793

Memili, E., & First, N. L. (2000). Zygotic and embryonic gene expression in cow: A review of timing and mechanisms of early gene expression as compared with other species. *Zygote*, *8*(1), 87–96. https://doi.org/10.1017/S0967199400000861

Menchero, S., Rayon, T., Andreu, M. J., & Manzanares, M. (2017). Signaling pathways in mammalian preimplantation development: Linking cellular phenotypes to lineage decisions. *Developmental Dynamics*, *246*(4), 245–261. https://doi.org/10.1002/dvdy.24471

Ménézo, Y. J., & Hérubel, F. (2002). Mouse and bovine models for human IVF*. *Reproductive BioMedicine Online*, *4*(2), 170–175. https://doi.org/10.1016/S1472-6483(10)61936-0

Ménézo, Y., Veiga, A., & Benkhalifa, M. (1998). Improved methods for blastocyst formation and culture. *Human Reproduction (Oxford, England)*, *13 Suppl 4*, 256–265. https://doi.org/10.1093/humrep/13.suppl_4.256

Meyuhas, O. (2015). Chapter Two - Ribosomal Protein S6 Phosphorylation: Four Decades of Research. In K. W. Jeon (Ed.), *International Review of Cell and Molecular Biology* (Vol. 320, pp. 41–73). Academic Press. https://doi.org/10.1016/bs.ircmb.2015.07.006

Milazzotto, M. P., Feitosa, W. B., Paula-Lopes, F. F., Buratini, J., Visintin, J. A., & Assumpção, M. E. O. A. (2012). The Mechanism of Oocyte Activation Influences the Cell Cycle–Related Genes Expression During Bovine Preimplantation Development. *Cellular Reprogramming*, *14*(5), 418–424. https://doi.org/10.1089/cell.2012.0024

Miller, D. J., Eckert, J. J., Lazzari, G., Duranthon-Richoux, V., Sreenan, J., Morris, D., Galli, C., Renard, J.-P., & Fleming, T. P. (2003). Tight junction messenger RNA expression levels in bovine embryos are dependent upon the ability to compact and in vitro culture methods. *Biology of Reproduction*, *68*(4), 1394–1402. https://doi.org/10.1095/biolreprod.102.009951

Mingoti, G. Z, Garcia, J. M., & Rosa-e-Silva, A. A. M. (2002). Steroidogenesis in cumulus cells of bovine cumulus–oocyte-complexes matured in vitro with BSA and different concentrations of steroids. *Animal Reproduction Science*, *69*(3), 175–186. https://doi.org/10.1016/S0378-4320(01)00187-7

Mingoti, Gisele Zoccal, Castro, V. S. D. C., Méo, S. C., Sá Barretto, L. S., & Garcia, J. M. (2011). The effects of macromolecular and serum supplements and oxygen tension during bovine in vitro procedures on kinetics of oocyte maturation and embryo development. *In Vitro Cellular & Developmental Biology. Animal*, *47*(5–6), 361–367. https://doi.org/10.1007/s11626-011-9400-0

Mishra, A., Reddy, I., Gupta, P., & Mondal, S. (2016). L-carnitine mediated reduction in oxidative stress and alteration in transcript level of antioxidant enzymes in sheep embryos produced in vitro. *Reproduction in Domestic Animals*, *51*(2), 311–321. https://doi.org/10.1111/rda.12682

Miyata, Y., Sato, T., Imada, K., Dobashi, A., Yano, M., & Ito, A. (2008). A citrus polymethoxyflavonoid, nobiletin, is a novel MEK inhibitor that exhibits antitumor metastasis in human fibrosarcoma HT-1080 cells. *Biochemical and Biophysical Research Communications*, *366*(1), 168–173. https://doi.org/10.1016/j.bbrc.2007.11.100

Miyata, Y., Sato, T., Yano, M., & Ito, A. (2004). Activation of protein kinase C betaII/epsilon-c-Jun NH2-terminal kinase pathway and inhibition of mitogen-activated protein/extracellular signal-regulated kinase 1/2 phosphorylation in antitumor invasive activity induced by the polymethoxy flavonoid, nobiletin. *Molecular Cancer Therapeutics*, *3*(7), 839–847.

Mnatsakanyan, H., Sabater i Serra, R., Salmeron-Sanchez, M., & Rico, P. (2019). Zinc Maintains Embryonic Stem Cell Pluripotency and Multilineage Differentiation Potential via AKT Activation. *Frontiers in Cell and Developmental Biology*, *7*. https://doi.org/10.3389/fcell.2019.00180

Mo, X., Wu, G., Yuan, D., Jia, B., Liu, C., Zhu, S., & Hou, Y. (2014). Leukemia inhibitory factor enhances bovine oocyte maturation and early embryo development. *Molecular Reproduction and Development*, *81*(7), 608–618. https://doi.org/10.1002/mrd.22327

Moore, K., Rodríguez-Sallaberry, C. J., Kramer, J. M., Johnson, S., Wroclawska, E., Goicoa, S., & Niasari-Naslaji, A. (2007). In vitro production of bovine embryos in medium supplemented with a serum replacer: Effects on blastocyst development, cryotolerance and survival to term. *Theriogenology*, *68*(9), 1316–1325. https://doi.org/10.1016/j.theriogenology.2007.08.034

Moosavi, F., Hosseini, R., Saso, L., & Firuzi, O. (2015). Modulation of neurotrophic signaling pathways by polyphenols. *Drug Design, Development and Therapy*, *10*, 23–42. https://doi.org/10.2147/DDDT.S96936

Morales, H., Tilquin, P., Rees, J. F., Massip, A., Dessy, F., & Van Langendonckt, A. (1999). Pyruvate prevents peroxide-induced injury of in vitro preimplantation bovine embryos. *Molecular Reproduction and Development*, *52*(2), 149–157. https://doi.org/10.1002/(SICI)1098-2795(199902)52:2<149::AID-MRD5>3.0.CO;2-4

Morley, K. L., Ferguson, P. J., & Koropatnick, J. (2007). Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. *Cancer Letters*, *251*(1), 168–178. https://doi.org/10.1016/j.canlet.2006.11.016

Moussa, M., Yang, C.-Y., Zheng, H.-Y., Li, M.-Q., Yu, N.-Q., Yan, S.-F., Huang, J.-X., & Shang, J.-H. (2019). Vitrification alters cell adhesion related genes in pre-implantation buffalo embryos: Protective role of β-mercaptoethanol. *Theriogenology*, *125*, 317–323. https://doi.org/10.1016/j.theriogenology.2018.11.013

Mukherjee, A., Malik, H., Saha, A. P., Dubey, A., Singhal, D. K., Boateng, S., Saugandhika, S., Kumar, S., De, S., Guha, S. K., & Malakar, D. (2014). Resveratrol treatment during goat oocytes maturation enhances developmental competence of parthenogenetic and hand-made cloned blastocysts by modulating intracellular glutathione level and embryonic gene expression. *Journal of Assisted Reproduction and Genetics*, *31*(2), 229–239. https://doi.org/10.1007/s10815-013-0116-9

Mulvihill, E. E., Assini, J. M., Lee, J. K., Allister, E. M., Sutherland, B. G., Koppes, J. B., Sawyez, C. G., Edwards, J. Y., Telford, D. E., Charbonneau, A., St-Pierre, P., Marette, A., & Huff, M. W. (2011). Nobiletin Attenuates VLDL Overproduction, Dyslipidemia, and Atherosclerosis in Mice With Diet-Induced Insulin Resistance. *Diabetes*, *60*(5), 1446–1457. https://doi.org/10.2337/db10-0589

Nakamura, T., Iwase, A., Bayasula, B., Nagatomo, Y., Kondo, M., Nakahara, T., Takikawa, S., Goto, M., Kotani, T., Kiyono, T., & Kikkawa, F. (2015). CYP51A1 induced by growth differentiation factor 9 and follicle-stimulating hormone in granulosa cells is a possible predictor for unfertilization. *Reproductive Sciences*, *22*(3), 377–384. https://doi.org/10.1177/1933719114529375

Natale, D. R., Paliga, A. J. M., Beier, F., D'Souza, S. J. A., & Watson, A. J. (2004). P38 MAPK signaling during murine preimplantation development. *Developmental Biology*, *268*(1), 76–88. https://doi.org/10.1016/j.ydbio.2003.12.011

Nguyen, C. N., Kim, H.-E., & Lee, S.-G. (2013). Caffeoylserotonin Protects Human Keratinocyte HaCaT Cells against H2O2-Induced Oxidative Stress and Apoptosis through Upregulation of HO-1 Expression via Activation of the PI3K/Akt/Nrf2 Pathway. *Phytotherapy Research*, *27*(12), 1810–1818. https://doi.org/10.1002/ptr.4931

Niemann, H., & Wrenzycki, C. (2000). Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: Implications for subsequent development. *Theriogenology*, *53*(1), 21–34. https://doi.org/10.1016/S0093-691X(99)00237-X

Niwa, H., Toyooka, Y., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, R., & Rossant, J. (2005). Interaction between Oct3/4 and Cdx2 Determines Trophectoderm Differentiation. *Cell*, *123*(5), 917–929. https://doi.org/10.1016/j.cell.2005.08.040

Noguchi, S., Atsumi, H., Iwao, Y., Kan, T., & Itai, S. (2016). Nobiletin: A citrus flavonoid displaying potent physiological activity. *Acta Crystallographica Section C: Structural Chemistry*, *72*(2), 124–127. https://doi.org/10.1107/S2053229616000577

Onda, K., Horike, N., Suzuki, T., & Hirano, T. (2013). Polymethoxyflavonoids Tangeretin and Nobiletin Increase Glucose Uptake in Murine Adipocytes. *Phytotherapy Research*, *27*(2), 312–316. https://doi.org/10.1002/ptr.4730

O'Neill, C. (2008). Phosphatidylinositol 3-kinase signaling in mammalian preimplantation embryo development. *Reproduction (Cambridge, England)*, *136*(2), 147–156. https://doi.org/10.1530/REP-08-0105

O'Shea, L. C., Mehta, J., Lonergan, P., Hensey, C., & Fair, T. (2012). Developmental competence in oocytes and cumulus cells: Candidate genes and networks. *Systems Biology in Reproductive Medicine*, *58*(2), 88–101. https://doi.org/10.3109/19396368.2012.656217

Padayatty, S. J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.-H., Chen, S., Corpe, C., Dutta, A., Dutta, S. K., & Levine, M. (2003). Vitamin C as an antioxidant: Evaluation of its role in disease prevention. *Journal of the American College of Nutrition*, *22*(1), 18–35. https://doi.org/10.1080/07315724.2003.10719272

Palma, G. A., Argañaraz, M. E., Barrera, A. D., Rodler, D., Mutto, A. Á., & Sinowatz, F. (2012). *Biology and Biotechnology of Follicle Development* [Review Article]. The Scientific World Journal; Hindawi. https://doi.org/10.1100/2012/938138

Pamplona, R., & Costantini, D. (2011). Molecular and structural antioxidant defenses against oxidative stress in animals. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *301*(4), R843–R863. https://doi.org/10.1152/ajpregu.00034.2011

Pang, Y., Zhao, S., Sun, Y., Jiang, X., Hao, H., Du, W., & Zhu, H. (2018). Protective effects of melatonin on the in vitro developmental competence of bovine oocytes. *Animal Science Journal*, *89*(4), 648–660. https://doi.org/10.1111/asj.12970

Panić, L., Tamarut, S., Sticker-Jantscheff, M., Barkić, M., Solter, D., Uzelac, M., Grabušić, K., & Volarević, S. (2006). Ribosomal Protein S6 Gene Haploinsufficiency Is Associated with Activation of a p53-Dependent Checkpoint during Gastrulation. *Molecular and Cellular Biology*, *26*(23), 8880–8891. https://doi.org/10.1128/MCB.00751-06

Parrish, J. J. (2014). Bovine in vitro fertilization: In vitro oocyte maturation and sperm capacitation with heparin. *Theriogenology*, *81*(1), 67–73. https://doi.org/10.1016/j.theriogenology.2013.08.005

Pérez-Pastén, R., Martínez-Galero, E., & Chamorro-Cevallos, G. (2010). Quercetin and naringenin reduce abnormal development of mouse embryos produced by hydroxyurea. *The Journal of Pharmacy and Pharmacology*, *62*(8), 1003–1009. https://doi.org/10.1111/j.2042-7158.2010.01118.x

Perkel, K. J., Tscherner, A., Merrill, C., Lamarre, J., & Madan, P. (2015). The ART of selecting the best embryo: A review of early embryonic mortality and bovine embryo viability assessment methods. *Molecular Reproduction and Development*, *82*(11), 822–838. https://doi.org/10.1002/mrd.22525

Picton, H., Briggs, D., & Gosden, R. (1998). The molecular basis of oocyte growth and development. *Molecular and Cellular Endocrinology*, *145*(1), 27–37. https://doi.org/10.1016/S0303-7207(98)00166-X

Plotnikov, A., Zehorai, E., Procaccia, S., & Seger, R. (2011). The MAPK cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1813*(9), 1619–1633. https://doi.org/10.1016/j.bbamcr.2010.12.012

Preis, K. A., Seidel, G. E., & Gardner, D. K. (2007). Reduced oxygen concentration improves the developmental competence of mouse oocytes following in vitro maturation. *Molecular Reproduction and Development*, *74*(7), 893–903. https://doi.org/10.1002/mrd.20655

Qu, Y., Liu, Y., Chen, L., Zhu, Y., Xiao, X., Wang, D., & Zhu, Y. (2018). Nobiletin prevents cadmium-induced neuronal apoptosis by inhibiting reactive oxygen species and modulating JNK/ERK1/2 and Akt/mTOR networks in rats. *Neurological Research*, *40*(3), 211–220. https://doi.org/10.1080/01616412.2018.1424685

Quetglas, M. D., Adona, P. R., Bem, T. D., Pires, P. R. L., & Leal, C. L. V. (2010). Effect of Cyclin-dependent Kinase (CDK) inhibition on expression, localization and activity of maturation promoting factor (MPF) and mitogen activated protein kinase (MAPK) in bovine oocytes. *Reproduction in Domestic Animals*, *45*(6), 1074–1081. https://doi.org/10.1111/j.1439-0531.2009.01498.x

Ramalho-Santos, J., Varum, S., Amaral, S., Mota, P. C., Sousa, A. P., & Amaral, A. (2009). Mitochondrial functionality in reproduction: From gonads and gametes to embryos and embryonic stem cells. *Human Reproduction Update*, *15*(5), 553–572. https://doi.org/10.1093/humupd/dmp016

Raman, M., Chen, W., & Cobb, M. H. (2007). Differential regulation and properties of MAPKs. *Oncogene*, *26*(22), 3100–3112. https://doi.org/10.1038/sj.onc.1210392

Ramos-Ibeas, P., Heras, S., Gómez-Redondo, I., Planells, B., Fernández-González, R., Pericuesta, E., Laguna-Barraza, R., Pérez-Cerezales, S., & Gutiérrez-Adán, A. (2019). Embryo responses to stress induced by assisted reproductive technologies. *Molecular Reproduction and Development*, *86*(10), 1292–1306. https://doi.org/10.1002/mrd.23119

Razzaghi-Asl, N., Garrido, J., Khazraei, H., Borges, F., & Firuzi, O. (2013). *Antioxidant Properties of Hydroxycinnamic Acids: A Review of Structure- Activity Relationships* [Text]. Bentham Science Publishers. https://www.ingentaconnect.com/content/ben/cmc/2013/00000020/00000036/art00002

Read, C. C., Willhelm, G., & Dyce, P. W. (2018). Connexin 43 coupling in bovine cumulus cells, during the follicular growth phase, and its relationship to in vitro embryo outcomes. *Molecular Reproduction and Development*, *85*(7), 579–589. https://doi.org/10.1002/mrd.22993

Renaud, S. J., Kubota, K., Rumi, M. A. K., & Soares, M. J. (2014). The FOS Transcription Factor Family Differentially Controls Trophoblast Migration and Invasion. *Journal of Biological Chemistry*, *289*(8), 5025–5039. https://doi.org/10.1074/jbc.M113.523746

Rieger, D., Loskutoff, N. M., & Betteridge, K. J. (1992). Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro. *Reproduction, Fertility, and Development*, *4*(5), 547–557. https://doi.org/10.1071/rd9920547

Riethmacher, D., Brinkmann, V., & Birchmeier, C. (1995). A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(3), 855–859. https://doi.org/10.1073/pnas.92.3.855

Riley, J. K., Carayannopoulos, M. O., H. Wyman, A., Chi, M., Ratajczak, C. K., & Moley, K. H. (2005). The PI3K/Akt pathway is present and functional in the preimplantation mouse embryo. *Developmental Biology*, *284*(2), 377–386. https://doi.org/10.1016/j.ydbio.2005.05.033

Rizos, D., Clemente, M., Bermejo-Alvarez, P., Fuente, J. D. L., Lonergan, P., & Gutiérrez-Adán, A. (2008). Consequences of In Vitro Culture Conditions on Embryo Development and Quality. *Reproduction in Domestic Animals*, *43*(s4), 44–50. https://doi.org/10.1111/j.1439-0531.2008.01230.x

Rizos, D., Gutiérrez-Adán, A., Pérez-Garnelo, S., De La Fuente, J., Boland, M. P., & Lonergan, P. (2003). Bovine embryo culture in the presence or absence of serum: Implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biology of Reproduction*, *68*(1), 236–243. https://doi.org/10.1095/biolreprod.102.007799

Rizos, D, Maillo, V., Sánchez-Calabuig, M.-J., & Lonergan, P. (2017). The Consequences of Maternal-Embryonic Cross Talk During the Periconception Period on Subsequent Embryonic Development. In A. Fazeli & W. V. Holt (Eds.), *Periconception in Physiology and Medicine* (pp. 69–86). Springer International Publishing. https://doi.org/10.1007/978-3-319-62414-3_4

Rizos, Dimitrios, Fair, T., Papadopoulos, S., Boland, M. P., & Lonergan, P. (2002). Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. *Molecular Reproduction and Development*, *62*(3), 320–327. https://doi.org/10.1002/mrd.10138

Rizos, Dimitrios, Ward, F., Duffy, P., Boland, M. P., & Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development*, *61*(2), 234–248. https://doi.org/10.1002/mrd.1153

Roberts, R., Franks, S., & Hardy, K. (2002). Culture environment modulates maturation and metabolism of human oocytes. *Human Reproduction (Oxford, England)*, *17*(11), 2950–2956. https://doi.org/10.1093/humrep/17.11.2950

Rocha, J. C., Passalia, F., Matos, F. D., Maserati Jr, M. P., Alves, M. F., de Almeida, T. G., Cardoso, B. L., Basso, A. C., & Nogueira, M. F. G. (2016). Methods for assessing the quality of mammalian embryos: How far we are from the gold standard? *JBRA Assisted Reproduction*, *20*(3), 150–158. https://doi.org/10.5935/1518-0557.20160033

Rocha-Frigoni, N. A. de S., Leão, B. C. da S., Nogueira, É., Accorsi, M. F., & Mingoti, G. Z. (2015). Effects of gaseous atmosphere and antioxidants on the development and cryotolerance of bovine embryos

at different periods of *in vitro* culture. *Zygote*, *23*(2), 159–168. https://doi.org/10.1017/S0967199413000361

Rocha-Frigoni, N. A. S., Leão, B. C. S., Dall'Acqua, P. C., & Mingoti, G. Z. (2016). Improving the cytoplasmic maturation of bovine oocytes matured in vitro with intracellular and/or extracellular antioxidants is not associated with increased rates of embryo development. *Theriogenology*, *86*(8), 1897–1905. https://doi.org/10.1016/j.theriogenology.2016.06.009

Roche, J. F. (1996). Control and regulation of folliculogenesis—A symposium in perspective. *Reviews of Reproduction*, *1*(1), 19–27. https://doi.org/10.1530/ror.0.0010019

Rodríguez, A., Diez, C., Ikeda, S., Royo, L. J., Caamaño, J. N., Alonso-Montes, C., Goyache, F., Alvarez, I., Facal, N., & Gomez, E. (2006). Retinoids during the in vitro transition from bovine morula to blastocyst. *Human Reproduction (Oxford, England)*, *21*(8), 2149–2157. https://doi.org/10.1093/humrep/del099

Rodriguez, K. F., & Farin, C. E. (2003). Gene transcription and regulation of oocyte maturation. *Reproduction, Fertility and Development*, *16*(2), 55–67. https://doi.org/10.1071/RD03078

Rose-Hellekant, T. A., Libersky-Williamson, E. A., & Bavister, B. D. (1998). Energy substrates and amino acids provided during in vitro maturation of bovine oocytes alter acquisition of developmental competence. *Zygote (Cambridge, England)*, *6*(4), 285–294. https://doi.org/10.1017/s0967199498000239

Ruvinsky, I., & Meyuhas, O. (2006). Ribosomal protein S6 phosphorylation: From protein synthesis to cell size. *Trends in Biochemical Sciences*, *31*(6), 342–348. https://doi.org/10.1016/j.tibs.2006.04.003

Sagirkaya, H., Misirlioglu, M., Kaya, A., First, N. L., Parrish, J. J., & Memili, E. (2006). Developmental and molecular correlates of bovine preimplantation embryos. *Reproduction (Cambridge, England)*, *131*(5), 895–904. https://doi.org/10.1530/rep.1.01021

Sakaguchi, K., Tanida, T., Abdel-Ghani, M. A., Kanno, C., Yanagawa, Y., Katagiri, S., & Nagano, M. (2018). Relationship between the antral follicle count in bovine ovaries from a local abattoir and steroidogenesis of granulosa cells cultured as oocyte-cumulus-granulosa complexes. *Journal of Reproduction and Development*, *advpub*. https://doi.org/10.1262/jrd.2018-080

Sakurai, N., Takahashi, K., Emura, N., Fujii, T., Hirayama, H., Kageyama, S., Hashizume, T., & Sawai, K. (2016). The Necessity of OCT-4 and CDX2 for Early Development and Gene Expression Involved in Differentiation of Inner Cell Mass and Trophectoderm Lineages in Bovine Embryos. *Cellular Reprogramming*, *18*(5), 309–318. https://doi.org/10.1089/cell.2015.0081

Salhab, M., Dhorne-Pollet, S., Auclair, S., Guyader-Joly, C., Brisard, D., Dalbies-Tran, R., Dupont, J., Ponsart, C., Mermillod, P., & Uzbekova, S. (2013). In vitro maturation of oocytes alters gene expression and signaling pathways in bovine cumulus cells. *Molecular Reproduction and Development*, *80*(2), 166–182. https://doi.org/10.1002/mrd.22148

Salzano, A., Albero, G., Zullo, G., Neglia, G., Abdel-Wahab, A., Bifulco, G., Zicarelli, L., & Gasparrini, B. (2014). Effect of resveratrol supplementation during culture on the quality and cryotolerance of bovine in vitro produced embryos. *Animal Reproduction Science*, *151*(3), 91–96. https://doi.org/10.1016/j.anireprosci.2014.09.018

Samardzija, M., Karadjole, M., Matkovic, M., Cergolj, M., Getz, I., Dobranic, T., Tomaskovic, A., Petric, J., Surina, J., Grizelj, J., & Karadjole, T. (2006). A comparison of BoviPure® and Percoll® on bull sperm separation protocols for IVF. *Animal Reproduction Science*, *91*(3), 237–247. https://doi.org/10.1016/j.anireprosci.2005.04.005

Sánchez, F., & Smitz, J. (2012). Molecular control of oogenesis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1822*(12), 1896–1912. https://doi.org/10.1016/j.bbadis.2012.05.013

Santiago-Moreno, J., Gómez-Brunet, A., González-Bulnes, A., Toledano-Díaz, A., Malpaux, B., & López-Sebastián, A. (2005). Differences in reproductive pattern between wild and domestic rams are not associated with inter-specific annual variations in plasma prolactin and melatonin concentrations. *Domestic Animal Endocrinology*, *28*(4), 416–429. https://doi.org/10.1016/j.domaniend.2005.02.002

Sasseville, M., Ritter, L. J., Nguyen, T. M., Liu, F., Mottershead, D. G., Russell, D. L., & Gilchrist, R. B. (2010). Growth differentiation factor 9 signaling requires ERK1/2 activity in mouse granulosa and cumulus cells. *Journal of Cell Science*, *123*(Pt 18), 3166–3176. https://doi.org/10.1242/jcs.063834

Sathananthan, A. H., Selvaraj, K., Girijashankar, M. L., Ganesh, V., Selvaraj, P., & Trounson, A. O. (2006). From oogonia to mature oocytes: Inactivation of the maternal centrosome in humans. *Microscopy Research and Technique*, *69*(6), 396–407. https://doi.org/10.1002/jemt.20299

Schindler, K. (2011). Protein Kinases and Protein Phosphatases that Regulate Meiotic Maturation in Mouse Oocytes. In J. Z. Kubiak (Ed.), *Cell Cycle in Development* (pp. 309–341). Springer. https://doi.org/10.1007/978-3-642-19065-0_14

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C T method. *Nature Protocols*, *3*(6), 1101–1108. https://doi.org/10.1038/nprot.2008.73

Sela-Abramovich, S., Chorev, E., Galiani, D., & Dekel, N. (2005). Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. *Endocrinology*, *146*(3), 1236–1244. https://doi.org/10.1210/en.2004-1006

Shi, M.-D., Liao, Y.-C., Shih, Y.-W., & Tsai, L.-Y. (2013). Nobiletin attenuates metastasis via both ERK and PI3K/Akt pathways in HGF-treated liver cancer HepG2 cells. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*, *20*(8–9), 743–752. https://doi.org/10.1016/j.phymed.2013.02.004

Simmet, K., Zakhartchenko, V., Philippou-Massier, J., Blum, H., Klymiuk, N., & Wolf, E. (2018). OCT4/POU5F1 is required for NANOG expression in bovine blastocysts. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(11), 2770–2775. https://doi.org/10.1073/pnas.1718833115

Singh, S. P., Wahajuddin, Tewari, D., Patel, K., & Jain, G. K. (2011). Permeability determination and pharmacokinetic study of nobiletin in rat plasma and brain by validated high-performance liquid chromatography method. *Fitoterapia*, *82*(8), 1206–1214. https://doi.org/10.1016/j.fitote.2011.08.010

Sirard, M. A. (2001). Resumption of meiosis: Mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology*, *55*(6), 1241–1254. https://doi.org/10.1016/S0093-691X(01)00480-0

Sirard, M.-A. (2012). Factors affecting oocyte and embryo transcriptomes. *Reproduction in Domestic Animals = Zuchthygiene*, *47 Suppl 4*, 148–155. https://doi.org/10.1111/j.1439-0531.2012.02069.x

Sirard, M.-A., Richard, F., & Mayes, M. (1998). Controlling meiotic resumption in bovine oocytes: A review. *Theriogenology*, *49*(2), 483–497. https://doi.org/10.1016/S0093-691X(97)00420-2

Sivanand, S., Viney, I., & Wellen, K. E. (2018). Spatiotemporal Control of Acetyl-CoA Metabolism in Chromatin Regulation. *Trends in Biochemical Sciences*, *43*(1), 61–74. https://doi.org/10.1016/j.tibs.2017.11.004

Smith, G. D. (2001). In vitro maturation of oocytes. *Current Women's Health Reports*, *1*(2), 143–151.

Somfai, T., Kaneda, M., Akagi, S., Watanabe, S., Haraguchi, S., Mizutani, E., Dang-Nguyen, T. Q., Geshi, M., Kikuchi, K., & Nagai, T. (2011). Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. *Reproduction, Fertility and Development*, *23*(7), 912. https://doi.org/10.1071/RD10339

Somfai, T., Ozawa, M., Noguchi, J., Kaneko, H., Kuriani Karja, N. W., Farhudin, M., Dinnyés, A., Nagai, T., & Kikuchi, K. (2007). Developmental competence of in vitro-fertilized porcine oocytes after in vitro maturation and solid surface vitrification: Effect of cryopreservation on oocyte antioxidative system and cell cycle stage. *Cryobiology*, *55*(2), 115–126. https://doi.org/10.1016/j.cryobiol.2007.06.008

Sovernigo, T. C., Adona, P. R., Monzani, P. S., Guemra, S., Barros, F. D. A., Lopes, F. G., & Leal, C. L. V. (2017). Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production. *Reproduction in Domestic Animals*, *52*(4), 561–569. https://doi.org/10.1111/rda.12946

Stojkovic, M., Machado, S. A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P. B., & Wolf, E. (2001). Mitochondrial Distribution and Adenosine Triphosphate Content of Bovine Oocytes Before and After In Vitro Maturation: Correlation with Morphological Criteria and Developmental Capacity After In Vitro Fertilization and Culture. *Biology of Reproduction*, *64*(3), 904–909. https://doi.org/10.1095/biolreprod64.3.904

Stokoe, D. (2005). The phosphoinositide 3-kinase pathway and cancer. *Expert Reviews in Molecular Medicine*, *7*(10), 1–22. https://doi.org/10.1017/S1462399405009361

Su, J.-D., Yen, J.-H., Li, S., Weng, C.-Y., Lin, M.-H., Ho, C.-T., & Wu, M.-J. (2012). 3',4'-didemethylnobiletin induces phase II detoxification gene expression and modulates PI3K/Akt signaling in PC12 cells. *Free Radical Biology & Medicine*, *52*(1), 126–141. https://doi.org/10.1016/j.freeradbiomed.2011.10.002

Su, Y.-Q., Sugiura, K., & Eppig, J. J. (2009). Mouse oocyte control of granulosa cell development and function: Paracrine regulation of cumulus cell metabolism. *Seminars in Reproductive Medicine*, *27*(1), 32–42. https://doi.org/10.1055/s-0028-1108008

Su, Y.-Q., Sugiura, K., Woo, Y., Wigglesworth, K., Kamdar, S., Affourtit, J., & Eppig, J. J. (2007). Selective degradation of transcripts during meiotic maturation of mouse oocytes. *Developmental Biology*, *302*(1), 104–117. https://doi.org/10.1016/j.ydbio.2006.09.008

Sun, Q.-Y., & Nagai, T. (2003). Molecular mechanisms underlying pig oocyte maturation and fertilization. *The Journal of Reproduction and Development*, *49*(5), 347–359. https://doi.org/10.1262/jrd.49.347

Sutton, M. L., Gilchrist, R. B., & Thompson, J. G. (2003). Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Human Reproduction Update*, *9*(1), 35–48. https://doi.org/10.1093/humupd/dmg009

Tadros, W., & Lipshitz, H. D. (2009). The maternal-to-zygotic transition: A play in two acts. *Development*, *136*(18), 3033–3042. https://doi.org/10.1242/dev.033183

Takahashi, M. (2012). Oxidative Stress and Redox Regulation on In Vitro Development of Mammalian Embryos. *Journal of Reproduction and Development*, *58*(1), 1–9. https://doi.org/10.1262/jrd.11-138N

Tao, Y., Chen, H., Tian, N. N., Huo, D. T., Li, G., Zhang, Y. H., Liu, Y., Fang, F. G., Ding, J. P., & Zhang, X. R. (2010). Effects of L-ascorbic acid, alpha-tocopherol and co-culture on in vitro developmental potential of porcine cumulus cells free oocytes. *Reproduction in Domestic Animals = Zuchthygiene*, *45*(1), 19–25. https://doi.org/10.1111/j.1439-0531.2008.01129.x

Tarazona, A. M., Rodríguez, J. I., Restrepo, L. F., & Olivera-Angel, M. (2006). Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced in vitro. *Reproduction in Domestic Animals*, *41*(1), 5–11. https://doi.org/10.1111/j.1439-0531.2006.00615.x

Tervit, H. R., Whittingham, D. G., & Rowson, L. E. A. (1972). SUCCESSFUL CULTURE IN VITRO OF SHEEP AND CATTLE OVA. *Reproduction*, *30*(3), 493–497. https://doi.org/10.1530/jrf.0.0300493

Tesfaye, D., Lonergan, P., Hoelker, M., Rings, F., Nganvongpanit, K., Havlicek, V., Besenfelder, U., Jennen, D., Tholen, E., & Schellander, K. (2007). Suppression of connexin 43 and E-cadherin transcripts in in vitro derived bovine embryos following culture in vitro or in vivo in the homologous bovine oviduct. *Molecular Reproduction and Development*, *74*(8), 978–988. https://doi.org/10.1002/mrd.20678

Tomek, W., Torner, H., & Kanitz, W. (2002). Comparative Analysis of Protein Synthesis, Transcription and Cytoplasmic Polyadenylation of mRNA during Maturation of Bovine Oocytes in vitro. *Reproduction in Domestic Animals*, *37*(2), 86–91. https://doi.org/10.1046/j.1439-0531.2002.00336.x

Tomek, Wolfgang, & Smiljakovic, T. (2005). Activation of Akt (protein kinase B) stimulates metaphase I to metaphase II transition in bovine oocytes. *Reproduction (Cambridge, England)*, *130*(4), 423–430. https://doi.org/10.1530/rep.1.00754

Torres, V., Hamdi, M., Blanca, M. M. de la, Urrego, R., Echeverri, J., López-Herrera, A., Rizos, D., Gutiérrez-Adán, A., & Sánchez-Calabuig, M. J. (2018). Resveratrol–cyclodextrin complex affects the

expression of genes associated with lipid metabolism in bovine in vitro produced embryos. *Reproduction in Domestic Animals*, *53*(4), 850–858. https://doi.org/10.1111/rda.13175

Torres, Viviana, Hamdi, M., Maillo, V., Urrego, R., Echeverri, J. J., López-Herrera, A., Gutiérrez-Adán, A., Rizos, D., & Sánchez-Calabuig, M. J. (2019). Ascorbic acid–cyclodextrin complex alters the expression of genes associated with lipid metabolism in bovine in vitro produced embryos. *Reproduction in Domestic Animals*, *54*(1), 55–62. https://doi.org/10.1111/rda.13311

Trigal, B., Gómez, E., Díez, C., Caamaño, J. N., Martín, D., Carrocera, S., & Muñoz, M. (2011). In vitro development of bovine embryos cultured with activin A. *Theriogenology*, *75*(3), 584–588. https://doi.org/10.1016/j.theriogenology.2010.09.010

Ufer, C., & Wang, C. C. (2011). The roles of glutathione peroxidases during embryo development. *Frontiers in Molecular Neuroscience*, *4*. https://doi.org/10.3389/fnmol.2011.00012

Ulbrich, S. E., Zitta, K., Hiendleder, S., & Wolf, E. (2010). In vitro systems for intercepting early embryo-maternal cross-talk in the bovine oviduct. *Theriogenology*, *73*(6), 802–816. https://doi.org/10.1016/j.theriogenology.2009.09.036

Van Hoeck, V., Leroy, J. L. M. R., Arias Alvarez, M., Rizos, D., Gutierrez-Adan, A., Schnorbusch, K., Bols, P. E. J., Leese, H. J., & Sturmey, R. G. (2013). Oocyte developmental failure in response to elevated nonesterified fatty acid concentrations: Mechanistic insights. *Reproduction (Cambridge, England)*, *145*(1), 33–44. https://doi.org/10.1530/REP-12-0174

van Loendersloot, L., van Wely, M., van der Veen, F., Bossuyt, P., & Repping, S. (2014). Selection of embryos for transfer in IVF: Ranking embryos based on their implantation potential using morphological scoring. *Reproductive Biomedicine Online*, *29*(2), 222–230. https://doi.org/10.1016/j.rbmo.2014.04.016

Van Soom, A., Boerjan, M. L., Bols, P. E., Vanroose, G., Lein, A., Coryn, M., & de Kruif, A. (1997). Timing of compaction and inner cell allocation in bovine embryos produced in vivo after superovulation. *Biology of Reproduction*, *57*(5), 1041–1049. https://doi.org/10.1095/biolreprod57.5.1041

Van Soom, A., Van Vlaenderen, I., Mahmoudzadeh, A. R., Deluyker, H., & de Kruif, A. (1992). Compaction rate of in vitro fertilized bovine embryos related to the interval from insemination to first cleavage. *Theriogenology*, *38*(5), 905–919. https://doi.org/10.1016/0093-691X(92)90165-N

Van Soom, A., Vanroose, G., & de Kruif, A. (2001). Blastocyst evaluation by means of differential staining: A practical approach. *Reproduction in Domestic Animals = Zuchthygiene*, *36*(1), 29–35. https://doi.org/10.1046/j.1439-0531.2001.00265.x

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., & Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. *Nature Reviews. Molecular Cell Biology*, *11*(5), 329–341. https://doi.org/10.1038/nrm2882

Viana, K. S., Caldas-Bussiere, M. C., Matta, S. G. C., Faes, M. R., Paes de Carvalho, C. S., & Quirino, C. R. (2007). Effect of sodium nitroprusside, a nitric oxide donor, on the in vitro maturation of bovine oocytes. *Animal Reproduction Science*, *102*(3), 217–227. https://doi.org/10.1016/j.anireprosci.2006.11.004

Wahli, W., & Michalik, L. (2012). PPARs at the crossroads of lipid signaling and inflammation. *Trends in Endocrinology & Metabolism*, *23*(7), 351–363. https://doi.org/10.1016/j.tem.2012.05.001

Wang, F., Tian, X., Zhang, L., He, C., Ji, P., Li, Y., Tan, D., & Liu, G. (2014). Beneficial effect of resveratrol on bovine oocyte maturation and subsequent embryonic development after in vitro fertilization. *Fertility and Sterility*, *101*(2), 577-586.e1. https://doi.org/10.1016/j.fertnstert.2013.10.041

Wang, S., Kou, Z., Jing, Z., Zhang, Y., Guo, X., Dong, M., Wilmut, I., & Gao, S. (2010). Proteome of mouse oocytes at different developmental stages. *Proceedings of the National Academy of Sciences*, *107*(41), 17639–17644. https://doi.org/10.1073/pnas.1013185107

Wang, X., Falcone, T., Attaran, M., Goldberg, J. M., Agarwal, A., & Sharma, R. K. (2002). Vitamin C and vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. *Fertility and Sterility*, *78*(6), 1272–1277. https://doi.org/10.1016/s0015-0282(02)04236-x

Watson, A. J., & Barcroft, L. C. (2001). Regulation of blastocyst formation. *Frontiers in Bioscience: A Journal and Virtual Library*, *6*, D708-730. https://doi.org/10.2741/watson

Webb, R. J., Marshall, F., Swann, K., & Carroll, J. (2002). Follicle-stimulating hormone induces a gap junction-dependent dynamic change in [cAMP] and protein kinase a in mammalian oocytes. *Developmental Biology*, *246*(2), 441–454. https://doi.org/10.1006/dbio.2002.0630

Wen, D., Noh, K.-M., Goldberg, A. D., Allis, C. D., Rosenwaks, Z., Rafii, S., & Banaszynski, L. A. (2014). Genome editing a mouse locus encoding a variant histone, H3.3B, to report on its expression in live animals: GENOME EDITING OF THE ENDOGENOUS H3.3B ALLELE. *Genesis*, *52*(12), 959–966. https://doi.org/10.1002/dvg.22827

Whitman, S. C., Kurowska, E. M., Manthey, J. A., & Daugherty, A. (2005). Nobiletin, a citrus flavonoid isolated from tangerines, selectively inhibits class A scavenger receptor-mediated metabolism of acetylated LDL by mouse macrophages. *Atherosclerosis*, *178*(1), 25–32. https://doi.org/10.1016/j.atherosclerosis.2004.07.034

Wolf, E., Arnold, G. J., Bauersachs, S., Beier, H. M., Blum, H., Einspanier, R., Fröhlich, T., Herrler, A., Hiendleder, S., Kölle, S., Prelle, K., Reichenbach, H.-D., Stojkovic, M., Wenigerkind, H., & Sinowatz, F. (2003). Embryo-Maternal Communication in Bovine – Strategies for Deciphering a Complex Cross-Talk. *Reproduction in Domestic Animals*, *38*(4), 276–289. https://doi.org/10.1046/j.1439-0531.2003.00435.x

Wrenzycki, C., & Stinshoff, H. (2013). Maturation Environment and Impact on Subsequent Developmental Competence of Bovine Oocytes. *Reproduction in Domestic Animals*, *48*(s1), 38–43. https://doi.org/10.1111/rda.12204

Wrenzycki, Christine. (2018). Gene expression analysis and in vitro production procedures for bovine preimplantation embryos: Past highlights, present concepts and future prospects. *Reproduction in Domestic Animals*, *53*(S2), 14–19. https://doi.org/10.1111/rda.13260

Wrenzycki, Christine, Herrmann, D., Lucas-Hahn, A., Korsawe, K., Lemme, E., & Niemann, H. (2004). Messenger RNA expression patterns in bovine embryos derived from in vitro procedures and their implications for development. *Reproduction, Fertility and Development*, *17*(2), 23–35. https://doi.org/10.1071/RD04109

Wu, G., & Schöler, H. R. (2014). Role of Oct4 in the early embryo development. *Cell Regeneration*, *3*(1). https://doi.org/10.1186/2045-9769-3-7

Wu, G.-Q., Jia, B.-Y., Li, J.-J., Fu, X.-W., Zhou, G.-B., Hou, Y.-P., & Zhu, S.-E. (2011). L-carnitine enhances oocyte maturation and development of parthenogenetic embryos in pigs. *Theriogenology*, *76*(5), 785–793. https://doi.org/10.1016/j.theriogenology.2011.04.011

Wu, X., Song, M., Gao, Z., Sun, Y., Wang, M., Li, F., Zheng, J., & Xiao, H. (2017). Nobiletin and its colonic metabolites suppress colitis-associated colon carcinogenesis by down-regulating iNOS, inducing antioxidative enzymes and arresting cell cycle progression. *The Journal of Nutritional Biochemistry*, *42*, 17–25. https://doi.org/10.1016/j.jnutbio.2016.12.020

Wu, X., Song, M., Rakariyatham, K., Zheng, J., Guo, S., Tang, Z., Zhou, S., & Xiao, H. (2015). Anti-inflammatory effects of 4′-demethylnobiletin, a major metabolite of nobiletin. *Journal of Functional Foods*, *19*, 278–287. https://doi.org/10.1016/j.jff.2015.09.035

Xu, S., Pang, L., Liu, Y., Lian, X., Mo, K., Lv, R., Zhu, H., Lv, C., Lin, J., Sun, J., Xu, L., & Wang, S. (2019). Akt plays indispensable roles during the first cell lineage differentiation of mouse. *Journal of Molecular Histology*, *50*(4), 369–374. https://doi.org/10.1007/s10735-019-09833-z

Yasuda, N., Ishii, T., Oyama, D., Fukuta, T., Agato, Y., Sato, A., Shimizu, K., Asai, T., Asakawa, T., Kan, T., Yamada, S., Ohizumi, Y., & Oku, N. (2014). Neuroprotective effect of nobiletin on cerebral ischemia–reperfusion injury in transient middle cerebral artery-occluded rats. *Brain Research*, *1559*, 46–54. https://doi.org/10.1016/j.brainres.2014.02.007

Ye, J., Li, J., Yu, Y., Wei, Q., Deng, W., & Yu, L. (2010). L-carnitine attenuates oxidant injury in HK-2 cells via ROS-mitochondria pathway. *Regulatory Peptides*, *161*(1–3), 58–66. https://doi.org/10.1016/j.regpep.2009.12.024

Yoshimizu, N., Otani, Y., Saikawa, Y., Kubota, T., Yoshida, M., Furukawa, T., Kumai, K., Kameyama, K., Fujii, M., Yano, M., Sato, T., Ito, A., & Kitajima, M. (2004). Anti-tumour effects of nobiletin, a citrus flavonoid, on gastric cancer include: Antiproliferative effects, induction of apoptosis and cell cycle deregulation. *Alimentary Pharmacology & Therapeutics*, *20*(s1), 95–101. https://doi.org/10.1111/j.1365-2036.2004.02082.x

Young, L., Sinclair, K., & Wilmut, I. (1998). Large offspring syndrome in cattle and sheep. *Reviews of Reproduction*, *3*(3), 155–163. https://doi.org/10.1530/ror.0.0030155

Yu, S., Long, H., Lyu, Q., Zhang, Q., Yan, Z., Liang, H., Chai, W., Yan, Z., Kuang, Y., & Qi, C. (2014a). Protective effect of quercetin on the development of preimplantation mouse embryos against hydrogen peroxide-induced oxidative injury. *PloS One*, *9*(2), e89520. https://doi.org/10.1371/journal.pone.0089520

Yu, S., Long, H., Lyu, Q., Zhang, Q., Yan, Z., Liang, H., Chai, W., Yan, Z., Kuang, Y., & Qi, C. (2014b). Protective Effect of Quercetin on the Development of Preimplantation Mouse Embryos against Hydrogen Peroxide-Induced Oxidative Injury. *PLoS ONE*, *9*(2). https://doi.org/10.1371/journal.pone.0089520

Yuan, Y. Q., Van Soom, A., Coopman, F. O. J., Mintiens, K., Boerjan, M. L., Van Zeveren, A., de Kruif, A., & Peelman, L. J. (2003). Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured in vitro. *Theriogenology*, *59*(7), 1585–1596. https://doi.org/10.1016/s0093-691x(02)01204-9

Zhang, D., Chen, H.-P., Duan, H.-F., Gao, L.-H., Shao, Y., Chen, K.-Y., Wang, Y.-L., Lan, F.-H., & Hu, X.-W. (2016). Aggregation of Ribosomal Protein S6 at Nucleolus Is Cell Cycle-Controlled and Its Function in Pre-rRNA Processing Is Phosphorylation Dependent. *Journal of Cellular Biochemistry*, *117*(7), 1649–1657. https://doi.org/10.1002/jcb.25458

Zhang, K., Wang, H., Rajput, S. K., Folger, J. K., & Smith, G. W. (2018). Characterization of H3.3 and HIRA expression and function in bovine early embryos. *Molecular Reproduction and Development*, *85*(2), 106–116. https://doi.org/10.1002/mrd.22939

Zhang, L., Zhang, X., Zhang, C., Bai, X., Zhang, J., Zhao, X., Chen, L., Wang, L., Zhu, C., Cui, L., Chen, R., Zhao, T., & Zhao, Y. (2016). Nobiletin promotes antioxidant and anti-inflammatory responses and elicits protection against ischemic stroke in vivo. *Brain Research*, *1636*, 130–141. https://doi.org/10.1016/j.brainres.2016.02.013

Zhang, T., Fan, X., Li, R., Zhang, C., & Zhang, J. (2018). Effects of pre-incubation with C-type natriuretic peptide on nuclear maturation, mitochondrial behavior, and developmental competence of sheep oocytes. *Biochemical and Biophysical Research Communications*, *497*(1), 200–206. https://doi.org/10.1016/j.bbrc.2018.02.054

Zhang, W., & Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research*, *12*(1), 9–18. https://doi.org/10.1038/sj.cr.7290105

Zhang, Y., Yang, Z., & Wu, J. (2007a). Signaling pathways and preimplantation development of mammalian embryos. *The FEBS Journal*, *274*(17), 4349–4359. https://doi.org/10.1111/j.1742-4658.2007.05980.x

Zhang, Y., Yang, Z., & Wu, J. (2007b). Signaling pathways and preimplantation development of mammalian embryos. *The FEBS Journal*, *274*(17), 4349–4359. https://doi.org/10.1111/j.1742-4658.2007.05980.x

Zhao, X.-M., Du, W.-H., Wang, D., Hao, H.-S., Liu, Y., Qin, T., & Zhu, H.-B. (2011). Recovery of mitochondrial function and endogenous antioxidant systems in vitrified bovine oocytes during extended in vitro culture. *Molecular Reproduction and Development*, *78*(12), 942–950. https://doi.org/10.1002/mrd.21389

Zhao, X.-M., Wang, N., Hao, H.-S., Li, C.-Y., Zhao, Y.-H., Yan, C.-L., Wang, H.-Y., Du, W.-H., Wang, D., Liu, Y., Pang, Y.-W., & Zhu, H.-B. (2018). Melatonin improves the fertilization capacity and developmental ability of bovine oocytes by regulating cytoplasmic maturation events. *Journal of Pineal Research*, *64*(1), e12445. https://doi.org/10.1111/jpi.12445

Zhong, R., & Zhou, D. (2013). Oxidative Stress and Role of Natural Plant Derived Antioxidants in Animal Reproduction. *Journal of Integrative Agriculture*, *12*(10), 1826–1838. https://doi.org/10.1016/S2095-3119(13)60412-8

Zhou, W., Nie, Z.-W., Zhou, D.-J., & Cui, X.-S. (2021). Acetyl-CoA synthases are essential for maintaining histone acetylation under metabolic stress during zygotic genome activation in pigs. *Journal of Cellular Physiology*, *n/a*. https://doi.org/10.1002/jcp.30355

Zou, W., Chen, C., Zhong, Y., An, J., Zhang, X., Yu, Y., Yu, Z., & Fu, J. (2013). PI3K/Akt Pathway Mediates Nrf2/ARE Activation in Human L02 Hepatocytes Exposed to Low-Concentration HBCDs. *Environmental Science & Technology*, *47*(21), 12434–12440. https://doi.org/10.1021/es401791s

Zuelke, K. A., & Bracketf, B. G. (1990). Luteinizing hormone-enhanced in vitro maturation of bovine oocytes with and without protein supplementation. *Biology of Reproduction*, *43*(5), 784–787. https://doi.org/10.1095/biolreprod43.5.784

Zullo, G., Albero, G., Neglia, G., De Canditiis, C., Bifulco, G., Campanile, G., & Gasparrini, B. (2016). L-ergothioneine supplementation during culture improves quality of bovine in vitro–produced embryos. *Theriogenology*, *85*(4), 688–697. https://doi.org/10.1016/j.theriogenology.2015.10.008

Zullo, G., De Canditiis, C., Pero, M. E., Albero, G., Salzano, A., Neglia, G., Campanile, G., & Gasparrini, B. (2016a). Crocetin improves the quality of in vitro–produced bovine embryos: Implications for blastocyst development, cryotolerance, and apoptosis. *Theriogenology*, *86*(8), 1879–1885. https://doi.org/10.1016/j.theriogenology.2016.06.011

Zullo, G., De Canditiis, C., Pero, M. E., Albero, G., Salzano, A., Neglia, G., Campanile, G., & Gasparrini, B. (2016b). Crocetin improves the quality of in vitro–produced bovine embryos: Implications for blastocyst development, cryotolerance, and apoptosis. *Theriogenology*, *86*(8), 1879–1885. https://doi.org/10.1016/j.theriogenology.2016.06.011

Curriculum Vitae

YULIA NATHALY CAJAS SUÁREZ

CONTACT

Phone:
+34 666093403
+593 981541269

yncajas

WEBSITE:
https://www.researchgate.net/profile/Yulia-N-Cajas
https://orcid.org/0000-0001-9791-6733
www.linkedin.com/in/yulia-cajas-suárez-37816839

email:
yncajas@gmail.com

EDUCATION

University Complutense of Madrid
2017 - 2021
PhD in Biochemistry, Molecular Biology and Biomedicine. Dept. of Biochemistry and Molecular Biology
Madrid, Spain

University Complutense of Madrid
2014 - 2015
Master in Biochemistry, Molecular Biology and Biomedicine
Madrid, Spain

Technical Particular University of Loja
2007 - 2012
Bachelor Degree in Biology
Loja, Ecuador

RESEARCH EXPERIENCE

NATIONAL INSTITUTE FOR AGRICULTURAL AND FOOD RESEARCH AND TECHNOLOGY (INIA-Madrid-Spain)
Preimplantation Embryology Laboratory.
Department of Animal Reproduction (INIA).

PhD Student
20/06/2017 al 03/07/2021

Investigative activity:

- In vitro embryo production including: maturation, fertilization and embryo culture
- Embryo differential staining
- Media Preparation
- Embryo freezing
- Ovary selection and recovery at slaughterhouse
- RNA extraction for embryo samples to study gene expression by quantitative real time PCR

- Extracellular vesicles isolation
- Embryo grading
- Vitrification of embryos: Cryoloop, Cryotop
- Immunofluorescence technique, immunohistochemistry
- Protein isolation for western blot analysis
- Confocal microscopy analysis
- Embryo vitrification: Cryoloop, Cryotop
- Image processing technologies
- Statistical analysis
- Design of experiments, writing short communications and scientific articles
- Western blot technique, ELISA, RIA
- Differential embryonic cell count
- Image processing technologies
- Co-culture of oviduct cells and embryos
- Evaluation of nuclear and cytoplasmic maturation of oocytes
- Participation in national and international research conferences

UNIVERSITY COMPLUTENSE OF MADRID (MADRID-SPAIN)
Department of Physiology (Animal Physiology)
Veterinary Faculty.

Master student
15/09/2014 al 15/09/2015

Investigative activity:

- Guinea pig oocytes in vitro maturation
- Oocyte staining
- Oocyte grading
- Ovarian collection and transport
- Ovary fixation
- Immunohistochemistry of EGF-R in guinea pig ovaries.
- Quantification of the immunofluorescence intensity
- Analysis of samples in confocal microscopy
- Writing short communications
- Presentation of international research congresses

TECHNICAL PARTICULAR UNIVERSITY OF LOJA (LOJA-ECUADOR)
Biotechnology of animal reproduction laboratory.
Department of Biological Sciences

Laboratory technician
04/02/2014 al 31/01/2017

Investigative activity:

- Media preparation
- Selection and collection of ovaries from slaughterhouse
- Selection of oocytes
- Maturation, fertilization, in vitro culture of embryos
- Stains
- RNA extraction for gene expression studies (PCR and qPCR)
- Immunohistochemistry and immunocytochemistry
- Evaluation of embryo quality
- Freezing of embryos.

Educational activity:

- Teacher at the School of Environmental Management of the Biology subject
- Teacher at the School of Biochemistry and Pharmacy of the Laboratory Protocols subject

PUBLICATIONS IN INDEXED JOURNALS

1. Cañón-Beltrán K., **Cajas Yulia N.,** Peréz-Cerezales S., Leal C. L. V., Agirregoitia E., Gutierrez-Adán A., González M., Rizos D. Nobiletin enhances the development and quality of bovine embryos in vitro during two key periods of embryonic genome activation. Sci Rep 11, 11796 (2021). https://doi.org/10.1038/s41598-021-91158-7 Impact factor: 3.9 - Quartile 1.

2. Cañón-Beltrán K., Hamdi M., Mazzarella M., **Cajas Yulia N.,** Leal C.L.V, Gutiérrez-Adán A., González E.M., da Silveira J., Rizos D. (2021) Isolation, Characterization, and MicroRNA Analysis of Extracellular Vesicles from Bovine Oviduct and Uterine Fluids. In: Brevini T.A., Fazeli A., Turksen K. (eds) Next Generation Culture Platforms for Reliable In Vitro Models. Methods in Molecular Biology, vol 2273. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1246-0_16

3. Cañón-Beltrán K., Giraldo-Giraldo J., **Cajas Y.N.,** Beltrán-Breña P., Hidalgo C.O., Vásquez N., Leal C.L.V, Gutiérrez-Adán A., González E.M., Rizos D. Inhibiting diacylglycerol acyltransferase-1 reduces lipid biosynthesis in bovine blastocysts produced in vitro. Theriogenology. 2020. Volume 158, 2020, Pages 267-276, ISSN 0093-691X, https://doi.org/10.1016/j.theriogenology.2020.09.014 Impact factor: 2.1 - Quartile 1.

4. **Cajas, Y.N.;** Cañón-Beltrán, K.; Ladrón de Guevara, M.; Millán de la Blanca, M.G.; Ramos-Ibeas, P.; Gutiérrez-Adán, A.; Rizos, D.; González, E.M. (2020). Antioxidant Nobiletin Enhances Oocyte Maturation and Subsequent Embryo Development and Quality. International Journal of Molecular Sciences. 2020, 21, 5340 doi.org/10.3390/ijms21155340. https://www.mdpi.com/781216 Impact factor: 4.556 - Quartile 1.

5. **Cajas Yulia N,** Karina E Cañón-Beltrán, M Encina González, Dimitrios Rizos. (2019). Interacciones materno-embrionarias preimplantacionales en la especie bovina. Revista Ecuatoriana de Ciencia Animal, [S.l.], v. 3, n. 2, p. 39-68, jun. 2019. ISSN 2602-8220. http://revistaecuatorianadecienciaanimal.com/index.php/RECA/article/view/117

PUBLICATIONS UNDER REVIEW

1. **Cajas YN.,** Cañón-Beltrán K., Núñez-Puente C., Gutierrez-Adán A., González E., Agirregoitia E., Rizos D. Nobiletin-induced partial abrogation of deleterious effects of AKT inhibition on preimplantation bovine embryo development in vitro. Submitido en la revista Biology of Reproduction- May 2021. IF: 3.3 Q1

2. Hamdi M., Cañón-Beltrán K., Mazzarella R., **Cajas YN.,** L.V. Leal C., Gutierrez-Adan A:, González E., da Silveira J., Rizos D. Characterization and profiling analysis of bovine oviduct and uterine extracellular vesicles and their miRNA cargo through the estrous cycle. Submitido en la revista FASEB Journal- June 2021. IF: 4.9 Q1

3. Leal CLV, Cañón-Beltrán K., **Cajas YN.,** Yaryes A., Hamdi M., Millán de la Blanca MG., Beltrán-Breña P., Mazzarella R., da Silveira JC., Gutierrez-Adán A., González E., Rizos D. Extracellular vesicles from oviductal and uterine fluids in sequential in vitro culture improve quality of bovine embryos. Submitido en la revista Frontiers in Veterinary Science IF: 2.2 Q1

PUBLICATIONS IN PREPARATION

1. Karina Cañón-Beltrán, **Yulia N Cajas,** Raúl Fernández-González, Encina González, Rosa García-García, María Arias-Álvarez, Dimitrios Rizos, Alfonso Gutiérrez-Adán. Influence of different in vitro capacitation protocols on the quality and capacity of murine and bovine heterologous fertilization of guinea pig spermatozoa.

2. Karina Cañón-Beltrán, **Yulia N Cajas,** Raúl Fernández-González, Encina González, Rosa García-García, María Arias-Álvarez, Dimitrios Rizos, Alfonso Gutiérrez-Adán. Review: Extracellular vesicles in bovine reproduction: from gamete maturation to pregnancy

POSTERS PRESENTED AT INTERNATIONAL CONFERENCES

1. **Cajas YN.,** Cañón-Beltrán K., Leal C. L. V., Gutierrez-Adán A., González E., Rizos D. Nobiletin affects gene expression profiles of the ERK1/2 pathway in bovine embryos produced in vitro. 47th Annual Conference of the International Embryo Transfer Society (IETS Virtual Meeting). Lima, Perú. January 2021. Reproduction, Fertility and Development 33(2) 135-135 https://doi.org/10.1071/RDv33n2Ab56

2. Hamdi M., Mazzarella R., Cañón-Beltrán K., **Cajas YN.,** Leal L.V. C., Gutierrez-Adan A., González E., da Silveira J., Rizos D. Analysis of miRNA content of oviduct and uterine extracellular vesicles across the bovine estrous cycle. 47th Annual Conference of the International Embryo Transfer Society (IETS Virtual Meeting). Lima, Perú. January 2021. Reproduction, Fertility and Development 33(2) 125-125 https://doi.org/10.1071/RDv33n2Ab36

3. Leal C. L. V., Cañón-Beltrán K., **Cajas YN.,** Yaryes A., Beltrán-Breña P., Hamdi M., Gutiérrez-Adán A., González M. E., Rizos D. Extracellular vesicles from oviduct and uterus in sequential in vitro culture affects mitochondrial activity and lipid metabolism transcripts in bovine embryos. 47th Annual Conference of the International Embryo Transfer Society (IETS Virtual Meeting). Lima, Perú. January 2021. Reproduction, Fertility and Development 33(2) 133-133 https://doi.org/10.1071/RDv33n2Ab52

4. **Cajas YN.,** Cañón-Beltrán K., Leal C. L. V., González E., Gutierrez-Adán A., Rizos D. Nobiletin supplementation affects gene expression profiles of the Akt pathway in bovine embryos in vitro. 46th Annual Conference of the International Embryo Transfer Society (IETS). New York, EEUU. January 2020. Reproduction, Fertility and Development 32(2) 164-165 https://doi.org/10.1071/RDv32n2Ab77

5. Cañón-Beltrán K., Giraldo-Giraldo J., Beltran P., **Cajas YN.,** Vásquez N., Leal C. L. V., Gutiérrez-Adán A., González E., Rizos D. Inhibiting Diacylglycerol Acyltransferase-1 enzyme in bovine

embryos produced in vitro reduces their lipid content and improves cryotolerance. Anim Reprod. 2020;17(3)

6. Hamdi M., Cañon-Beltrán K., Mazzarella R., **Cajas YN.,** Leal C. L. V., Gutierrez-Adan A., González E., da Silveira J., Rizos D. Characterization and analysis of miRNA content of bovine oviduct and uterine extracellular vesicles across estrous cycle. Anim Reprod 2020;17(3)

7. Cañón-Beltrán K., Giraldo-Giraldo J., **Cajas YN.,** Vásquez N., Leal C. L. V., Gutiérrez-Adán A., González E., Rizos D. Expression of lipid metabolism-related genes in bovine embryos cultured in vitro with diacylglycerol acyltransferase-1 inhibitor. 46th Annual Conference of the International Embryo Transfer Society (IETS). New York, EEUU. January 2020. Reproduction, Fertility and Development 32(2) 163-164 https://doi.org/10.1071/RDv32n2Ab75

8. Leal C. L. V., Cañon-Beltrán K., **Cajas YN.,** Gallego P., Beltrán-Breña P., Hamdi M., González E., Rizos D. Extracellular vesicles from oviduct and uterus in sequential culture improve the quality of bovine embryos produced in vitro. 46th Annual Conference of the International Embryo Transfer Society (IETS). New York, EEUU. January 2020. Reproduction, Fertility and Development 32(2) 164-164 https://doi.org/10.1071/RDv32n2Ab76

9. **Cajas YN.,** Cañón-Beltrán K., Leal C. L. V., González E., Rizos D. Nobiletin supplementation prior to EGA improves development and quality of bovine blastocysts in vitro. Anim. Reprod., v.16, n.3, p.686, Jul./Sept. 2019. ISSN 1984-3143, online and 1806-9614.

10. Giraldo-Giraldo J., Cañón-Beltrán K., **Cajas YN.,** Vásquez N., Leal C. L. V., González E., Rizos D. Diacylglycerol acyltransferase-1 (DGAT1) improves in vitro embryo development and quality in bovine. (AERA) Reprod Dom Anim. 2019. 54: 106-134. doi:10.1111/rda.13549.

11. **Cajas YN.,** Cañón-Beltrán K., González E., Ramos-Ibeas P., Gutierrez-Adán A., Rizos D. Nobiletin enhances the quality of in vitro-matured bovine oocytes and blastocysts by altering the transcription of key developmental genes. 45th Annual Conference of the International Embryo Transfer Society (IETS). New Orleans, EEUU. January 2019. Reproduction, Fertility and Development 31(1) 212-212 https://doi.org/10.1071/RDv31n1Ab175

12. **Cajas YN.,** Cañón-Beltrán K., Ladrón de Guevara M., Millán de la Blanca M.G., González E., Rizos D. Nobiletin supplementation in maturation media enhances in vitro oocyte maturation and subsequent embryo development. Anim. Reprod., v.15, n.3, p.540, Jul./Sept. 2018

13. Cañón-Beltrán K., Garcia-García R. **Cajas-Suárez Y.,** Carrera R.; Lorenzo P., Arias AlvarezM. Effect of Insulin – Like Growth Factor I (IGF-I) on oocyte in vitro maturation, apoptosis and steroidogenesis of cumulus-oocyte complexes in the guinea pig model. Reprod Dom Anim, 2016. 51: 66-153. doi:10.1111/rda.12801.

14. Cañón-Beltrán K., **Cajas Y.N.,** Carrera R.; Lorenzo P., Rebollar P.G., García-García R. M., Arias-Alvarez M. In vitro maturation of guinea pig oocytes supplemented with Epidermal Growth Factor and Insulin-Like Growth Factor I" Anim. Reprod., v.13, n.3, p.637, Jul./Sept. 2016

15. Cañón-Beltrán K., **Cajas Y.N.,** García-García R. M., Lorenzo P. L., Carrera R., Rebollar P. G., Arias-Alvarez M. Effect of epidermal growth factor on nuclear and cytoplasmic in vitro maturation of guinea pig oocytes". Anim. Reprod., v.12, n.3, p.793, Jul./Sept. 2015

PARTICIPATION IN NATIONAL AND INTERNATIONAL CONGRESSES

1. 47th Annual International Conference of the International Society for Embryo Technology.

IETS – EEUU. 18 – 21 de enero de 2021
2. 36th Scientific Meeting of the European Embryo Transfer Society (AETE)– Irlanda. 10 – 11 de septiembre de 2020 / 16 horas
3. 35th Scientific Meeting of the European Embryo Transfer Society (AETE)– Murcia. 13 – 14 de septiembre de 2019 / 11 horas
4. 34th Scientific Meeting of the European Embryo Transfer Society (AETE)– Nantes, Francia 13 – 14 de septiembre de 2018 / 11 horas
5. 18th International Congress of Animal Reproduction. ICAR - Tours, France 26 – 30 de junio de 2016
6. Taller de Fotografía. Universidad Técnica Particular de Loja UTPL – Loja, Ecuador. 10 de mayo al 02 de junio de 2016
7. 31th Scientific Meeting of the European Embryo Transfer Society (AETE)– Gante, Bélgica. 11 – 13 de septiembre del 2015
8. XXXV Jornadas Nacionales de Biología y I Congreso Ecuatoriano de Mastozoología. Universidad Técnica Particular de Loja UTPL – Loja, Ecuador. 17 al 19 de noviembre de 2012
9. Ciclo de conferencias 2011 Bioética para la promoción de la vida. Universidad Técnica Particular de Loja UTPL – Loja, Ecuador. Abril – Agosto, 2011
10. Curso de "Micología General". Universidad Técnica Particular de Loja UTPL – Loja, Ecuador. 27 de septiembre al 12 de noviembre de 2010.
11. 5to. Seminario Internacional de Buiatría. Universidad Técnica Particular de Loja- UTPL. Loja, Ecuador 27 - 29 de mayo de 2010- 18 horas
12. I Jornadas Nacionales de Bioquímica y II Congreso de estudiantes de Química, Bioquímica y Farmacia del Ecuador, Universidad Técnica Particular de Loja- UTPL. Loja, Ecuador. 13 al 15 de enero de 2010.
13. XXXII Jornadas Nacionales de Biología. Universidad Técnica Particular de Loja- UTPL. Loja, Ecuador. 20 - 22 de noviembre de 2008.
14. Segunda Conferencia Científica de Orquídeas de los Andes. Universidad Técnica Particular de Loja- UTPL. Loja, Ecuador. 14 - 17 de noviembre de 2007.

RESEARCH PROJECTS

1. **Título del proyecto:** "Estudio de las condiciones de producción y conservación de Embriones in vitro, para la mejora de las técnicas de Reproducción asistida de bovinos"
   Entidad financiadora: UTPL.
   Entidades participantes: UTPL
   Duración: Enero 2015 hasta Enero 2016
   Rol: Participante del proyecto

2. **Título del proyecto:** "Optimización del sistema productivo de cuyes o cobayas para La mejora de la economía de las familias de origen indigna en las comunidades de Chuquiribamba (Loja, ecuador)
   Entidad financiadora: UCM
   Entidades participantes: UCM y UTPL
   Duración: Enero 2014 hasta Diciembre 2014
   Rol: Participante del proyecto componente UTPL, Técnico de laboratorio

3. **Título del proyecto:** "Modelo matemático para el proceso de la maduración ovocitaria in vitro de la especie de cavia porcellus"
   Entidad financiadora: UTPL
   Entidades participantes: UTPL
   Duración: Enero 2014 hasta Diciembre 2014
   Rol: Participante del proyecto

4. **Título del proyecto:** "Estudio de la maduración ovocitaria, capacitación espermática, fecundación y cultivo in vitro de embriones de la especie cavia porcellus (fase 1)"
   Entidad financiadora: UTPL
   Entidades participantes: UTPL y UCM
   Duración: Enero 2013 hasta Diciembre 2013
   Rol: Participante del proyecto

AWARDS AND SCHOLARSHIPS

1. Winner for poster competition in the 35rd Scientific Meeting of the European Embryo Transfer Society (AETE). Murcia, Spain 2019
2. Scholarship granted of academic excellence by National Secretary of Higher Education, Science, Technology and Innovation (SENESCYT) to do PhD degree. Ecuador 2017-2020.
3. Scholarship granted by Spanish Agency for International Development Cooperation (AECID) to do master degree. Madrid, Spain. 2014-2015.

PROFESIONAL REFERENCES

Dr. Dimitrios Rizos
Principal Investigator
National Institute for Agricultural Research and Technology (INIA) - Spain
Contact: +34 913474045
Email: drizos@inia.es

Dra. Cláudia Lima Verde Leal
Professor
University of Sao Pablo Pirassununga- Brazil
Contact: +55 19 99324-0448
Email: clvleal@usp.br

Dr. Alfonso Gutiérrez Adán
Director of the Animal Reproduction Department
National Institute for Agricultural Research and Technology (INIA) - Spain
Contact: +34 913473768
Email: agutierr@inia.es

Dra. Karina Cañón - Beltrán

Postdoctoral Researcher
National Institute for Agricultural Research and Technology (INIA) - Spain
Juan de Castellanos University Foundation (Tunja-Boyacá-Colombia)
Contact: +34 666093419
Email: kecanon@gmail.com

Annexes

International Journal of
Molecular Sciences

MDPI

Article

Antioxidant Nobiletin Enhances Oocyte Maturation and Subsequent Embryo Development and Quality

Yulia N. Cajas [1], Karina Cañón-Beltrán [1], Magdalena Ladrón de Guevara [1], María G. Millán de la Blanca [1], Priscila Ramos-Ibeas [1], Alfonso Gutiérrez-Adán [1], Dimitrios Rizos [1] and Encina M. González [2,*]

1 Department of Animal Reproduction, National Institute for Agriculture and Food Research and Technology (INIA), Avda. Puerta de Hierro, 28040 Madrid, Spain; yncajas@gmail.com (Y.N.C.); kecanon@gmail.com (K.C.-B.); magdalenaladron@gmail.com (M.L.d.G.); mgmillanb@gmail.com (M.G.M.d.l.B.); priscilaramosibeas@gmail.com (P.R.-I.); agutierr@inia.es (A.G.-A.); drizos@inia.es (D.R.)

2 Department of Anatomy and Embryology, Veterinary Faculty, Complutense University of Madrid (UCM), 28040 Madrid, Spain

* Correspondence: mengonza@ucm.es

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Abstract: Nobiletin is a polymethoxylated flavonoid isolated from citrus fruits with wide biological effects, including inhibition of reactive oxygen species (ROS) production and cell cycle regulation, important factors for oocyte in vitro maturation (IVM). Therefore, the objective of the present study was to evaluate the antioxidant activity of nobiletin during IVM on matured bovine oocyte quality (nuclear and cytoplasmic maturation; oocyte mitochondrial activity; intracellular ROS and glutathione (GSH) levels) and their developmental competence, steroidogenesis of granulosa cells after maturation, as well as quantitative changes of gene expression in matured oocytes, their cumulus cells, and resulting blastocysts. Bovine cumulus-oocyte complexes were in vitro matured in TCM-199 +10% fetal calf serum (FCS) and 10 ng/mL epidermal growth factor (EGF) (Control) supplemented with 10, 25, 50, or 100 μM of nobiletin (Nob10, Nob25, Nob50, and Nob100, respectively) or 0.1% dimethyl sulfoxide (CDMSO: vehicle for nobiletin dilution). A significantly higher percentage of matured oocytes in metaphase II was observed in Nob25 and Nob50 compared to other groups. Similarly, cleavage rate and cumulative blastocyst yield on Days 7 and 8 were significantly higher for Nob25 and Nob50 groups. Oocytes matured with 25 and 50 μM nobiletin showed a higher rate of migration of cortical granules and mitochondrial activity and a reduction in the ROS and GSH content in comparison with all other groups. This was linked to a modulation in the expression of genes related to metabolism (*CYP51A1*), communication (*GJA1*), apoptosis (*BCL2*), maturation (*BMP15* and *MAPK1*), and oxidative stress (*SOD2* and *CLIC1*). In conclusion, nobiletin offers a novel alternative for counteracting the effects of the increase in the production of ROS during IVM, improves oocyte nuclear and cytoplasmic maturation, and subsequent embryo development and quality in cattle.

Keywords: nobiletin; oocyte quality; antioxidant; ROS; cattle; in vitro

1. Introduction

In vitro maturation (IVM) of bovine oocytes is one of the most important processes for the development of other assisted reproductive techniques, such as in vitro production of embryos (IVP). The accomplishment of this technique requires successful IVM that involves nuclear, cytoplasmic, and molecular maturation, necessary for subsequent embryonic development [1]. Nevertheless, IVP of cattle embryos still has limitations, considering that not all the oocytes have the ability to develop into

a viable embryo after IVM [2], as the culture systems decrease the quality of these gametes [3]. One of the causes is the increase in the production of reactive oxygen species (ROS) caused by the oxygen tension at which IVM is performed [4]. Under normal conditions, the cell produces a ROS level that acts beneficially for tissue regeneration, intracellular redox regulation, and embryogenesis, but an excess of ROS can oxidize cellular molecules, such as lipids, carbohydrates, amino acids, and nucleic acids, modifying their functions and compromising cellular viability by producing lipid peroxidation, mitochondrial damage, and apoptosis [4]. The strategy to avoid the harmful effects caused by an excess of ROS is the use of a wide variety of antioxidants during IVM, such as Vitamin C that protects cells against ROS and acts as an inhibitor of lipid peroxidation [5], cysteamine, which increases intracellular glutathione (GSH) content that protects cells from the deleterious effects of oxidative stress [6] and catalase, which reduces the intracellular concentrations of ROS during IVM and the percentage of apoptotic cells [3]. Moreover, exogenous antioxidants can also act as signaling molecules in steroidogenesis and intracellular redox regulation during IVM [6–8]. In recent years, there have been promising results with compounds of natural origin, such as resveratrol [8] or quercetin [6]. However, it is not yet clear which antioxidant is the most efficient to support the development, production, and quality of bovine embryos.

Nobiletin, a class of polymethoxylated flavone identified from the citrus peel (chemically known as 5,6,7,8,3′,4′ hexamethoxyflavone), has drawn increasing attention since it is easily absorbed across the cytoplasmic membranes due to its structure and lipophilic nature [9,10]. Nobiletin interacts with several signaling pathways (ERK, PI3K/AKT, CREB) to promote survival in various cell lines [10,11]. Moreover, nobiletin has a broad range of biological effects, including cell cycle regulation [10], reduction of apoptosis [11,12] and antioxidation [13], important also for the success of oocyte IVM.

Thus, in this study, we aimed to evaluate the antioxidant activity of nobiletin during IVM on matured bovine oocyte quality and their developmental competence. The parameters evaluated were, (i) nuclear (meiotic progression to metaphase II (M-II)) and cytoplasmic maturation (cortical granules (CG) and mitochondrial distribution pattern), (ii) oocyte mitochondrial activity, and intracellular ROS and GSH levels (iii), steroidogenesis of granulosa cells (iv), oocyte developmental competence to blastocyst stage, and (v) quantitative changes of gene expression in matured oocytes, their cumulus cells (CCs) and produced blastocysts.

2. Results

2.1. Nobiletin Enhances Oocyte In Vitro Maturation and Reduces Oxidative Stress

When evaluating the effect of nobiletin on nuclear maturation, we observed that a concentration of 25 (87.0 ± 0.6%) and 50 μM (89.3 ± 0.4%) increased ($p < 0.05$) the percentage of oocytes reaching M-II compared to all other groups (Nob10: 72.9 ± 0.4%; Nob100: 71.5 ± 0.8%; Control: 71.7 ± 0.8%; and CDMSO: 70.5 ± 0.5%) (Table 1).

The migration of CG to the cortical region of the oocyte, as well as mitochondrial distribution and their activity, were used as indicators to analyze cytoplasmic maturation. In the assessment of the cortical granule distribution patterns oocytes matured in the presence of Nob25 (85.7 ± 0.3%) and Nob50 (89.9 ± 2.2%) displayed a higher incidence of migrated CG than oocytes in the Control (69.1 ± 1.1%), CDMSO (69.6 ± 0.9%), Nob10 (72.1 ± 1.0%) and Nob100 (71.2 ± 0.7%) groups ($p < 0.05$). The presence of oocytes with a partially migrated pattern was lower ($p < 0.05$) in Nob25 and Nob50 than all other groups. Similarly, the non-migrated pattern distribution of CG was lower ($p < 0.05$) for nobiletin groups compared to the Control group, while for CDMSO, Nob10 and Nob100 no differences were observed (Table 1). Representative images of CG distribution in matured oocytes are presented in Figure 1.

Table 1. In vitro maturation of bovine oocytes in the presence of the nobiletin.

Parameters Evaluated	Control	CDMSO	Nob10	Nob25	Nob50	Nob100
Nuclear maturation *n*	117	122	133	146	149	144
Matured (M-II) *n* (%)	84 (71.7 ± 0.8)^{b}	86 (70.5 ± 0.5)^{b}	97 (72.9 ± 0.4)^{b}	127 (87.0 ± 0.6)^{a}	133 (89.3 ± 0.4)^{a}	103 (71.5 ± 0.8)^{b}
Immature *n* (%)	33 (28.2 ± 0.7)^{a}	36 (29.5 ± 0.5)^{a}	36 (27.1 ± 0.4)^{a}	19 (12.9 ± 0.6)^{b}	16 (10.7 ± 0.4)^{b}	41 (28.4 ± 0.8)^{a}
Cytoplasmic Maturation						
Cortical Granules Distribution						
n	58	66	72	70	78	70
Migrated *n* (%)	40 (69.1 ± 1.1)^{b}	46 (69.6 ± 0.9)^{b}	52 (72.1 ± 1.0)^{b}	60 (85.7 ± 0.3)^{a}	70 (89.9 ± 2.2)^{a}	50 (71.2 ± 0.7)^{b}
Partially migrated *n* (%)	10 (17.2 ± 2.6)^{a}	12 (18.2 ± 1.7)^{a}	15 (20.9 ± 0.7)^{a}	7 (9.9 ± 1.6)^{b}	7 (8.8 ± 1.3)^{b}	15 (21.5 ± 0.6)^{a}
Non-migrated *n* (%)	8 (13.7 ± 1.9)^{a}	8 (12.2 ± 2.0)^{ac}	5 (6.9 ± 0.2)^{bc}	3 (4.4 ± 1.8)^{b}	1 (1.2 ± 1.2)^{b}	5 (7.3 ± 0.2)^{bc}
Mitochondrial Distribution						
n	59	56	61	76	71	74
Migrated *n* (%)	42 (71.3 ± 1.5)^{b}	39 (69.7 ± 1.0)^{b}	45 (73.7 ± 1.0)^{b}	66 (86.7 ± 0.6)^{a}	63 (88.9 ± 1.2)^{a}	53 (71.6 ± 0.5)^{b}
Partially migrated *n* (%)	10 (17.0 ± 0.5)^{a}	11 (19.6 ± 1.1)^{a}	11 (17.9 ± 1.0)^{a}	5 (6.7 ± 0.3)^{b}	7 (9.8 ± 1.5) ^{b}	13 (17.5 ± 1.5)^{a}
Non-migrated *n* (%)	7 (11.7 ± 1.8)^{a}	6 (10.8 ± 1.5)^{a}	5 (8.3 ± 0.4)^{a}	5 (6.6 ± 0.3)^{ab}	1 (1.3 ± 1.3)^{b}	8 (10.8 ± 1.7)^{a}

n: number of oocytes assigned per group. Control: oocytes cultured in the presence of synthetic oviductal fluid (SOF) and 5% fetal calf serum (FCS); CDMSO: oocytes cultured in the presence of SOF + 5% FCS supplemented with 0.01% DMSO; Nob10, Nob25, Nob50, Nob100 oocytes cultured in presence of SOF + 5% FCS supplemented with 10, 25, 50, and 100 μM nobiletin, respectively. Data are the mean ± SEM. Within lanes, values with different superscript letters differ significantly ($p < 0.05$).

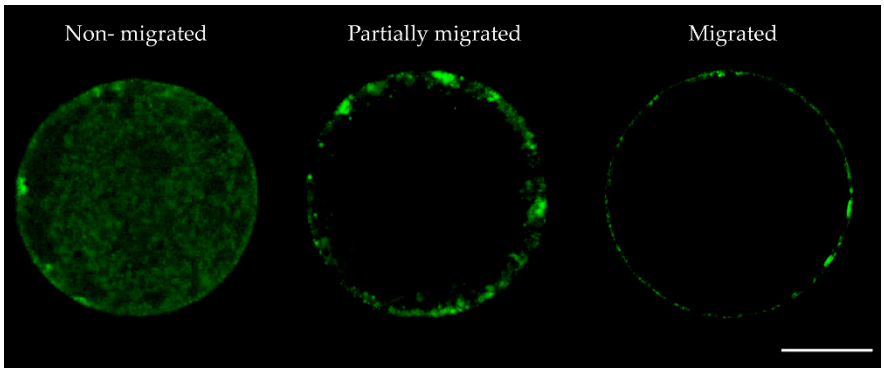

Figure 1. Representative fluorescent images of cortical granules (CG) distribution patterns in bovine oocytes after in vitro maturation in the presence of nobiletin. Scale bar 50 μm.

Regarding the mitochondrial distribution patterns, we found higher migration ($p < 0.05$) in oocytes matured with Nob25 (86.7 ± 0.6%) and Nob50 (88.9 ± 1.2%) compared to Control (71.3 ± 1.5%), CDMSO (69.7 ± 1.0%); Nob10 (73.7 ± 1.0%) and Nob100 (71.6 ± 0.5%) groups. The partially migrated mitochondrial pattern was lower ($p < 0.05$) in the oocytes matured with Nob25 and Nob50 compared to all other groups, while the incidence of non-migrated mitochondria pattern was lower ($p < 0.05$) only for Nob50 group (Table 1). Representative images of mitochondrial distribution in matured oocytes are presented in Figure 2. Quantification of mitochondrial activity in oocytes was measured by fluorescence intensity and a significant increase in intensity was observed in oocytes maturated with Nob25 and Nob50 compared to all other groups ($p < 0.05$; Supplementary Figure S1).

When evaluating the effect of nobiletin on oxidative stress, through a relative of ROS and GSH fluorescence intensity in maturated oocytes, we observed that the intensity in both parameters was lower ($p < 0.05$) in Nob25 and Nob50 groups compared with oocytes matured with Nob10 and Nob100 and control groups (Figure 3).

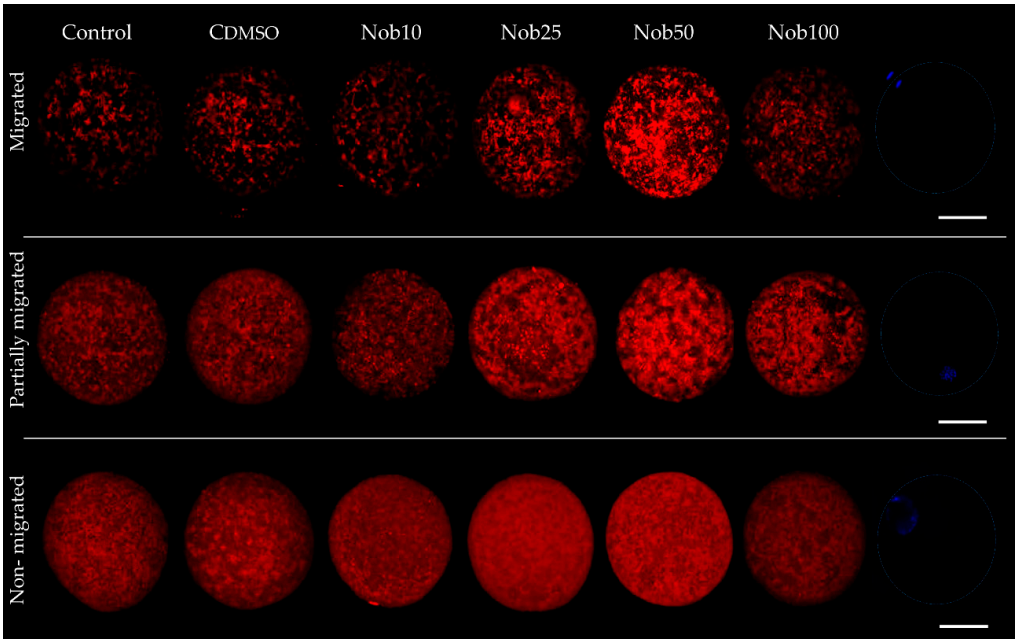

Figure 2. Representative fluorescent images of mitochondria migration pattern in bovine oocytes after in vitro maturation in the presence of nobiletin. Control: oocytes cultured in the presence of synthetic oviductal fluid (SOF) and 5% fetal calf serum (FCS); CDMSO: oocytes cultured in the presence of SOF + 5% FCS supplemented with 0.01% DMSO; Nob10, Nob25, Nob50, Nob100 oocytes cultured in presence of SOF + 5% FCS supplemented with 10, 25, 50 and 100 µM nobiletin, respectively. Scale bar 50 µm.

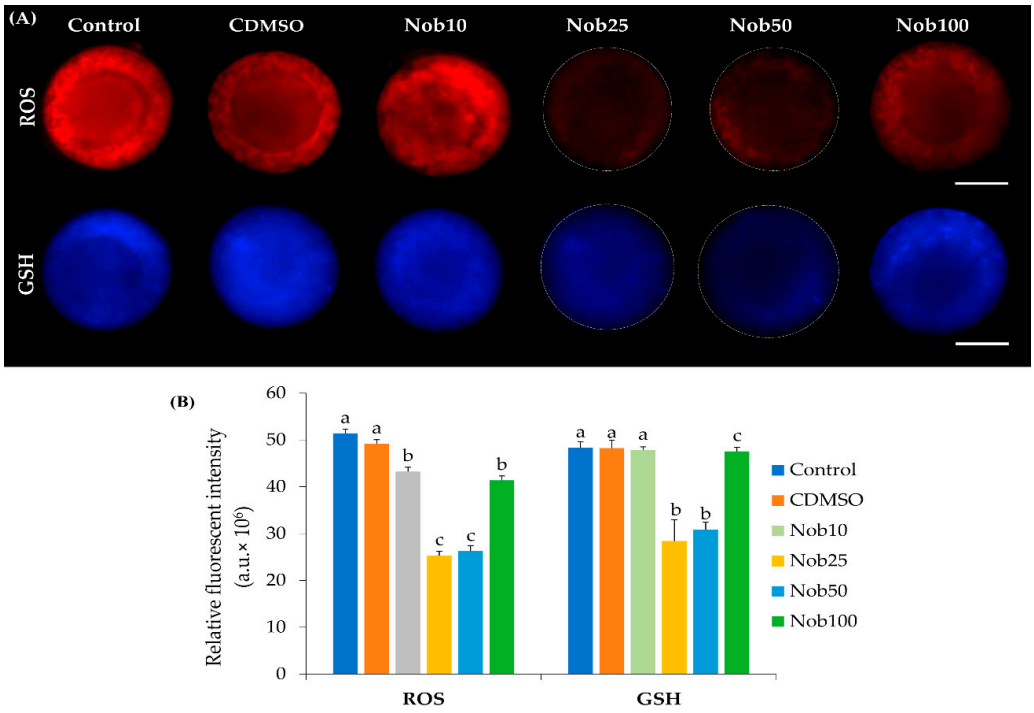

Figure 3. Reactive oxygen species (ROS) and glutathione (GSH) fluorescence intensity in bovine oocytes after in vitro maturation in the presence of nobiletin (**A**) Representative fluorescent images of ROS and GSH fluorescence intensity in bovine oocytes after in vitro maturation in the presence of nobiletin. Control (n = 54); CDMSO (n = 48); Nob10 (n = 50); Nob25 (n = 47); Nob50 (n = 53); Nob100 (n = 49). (**B**) Quantification of relative fluorescent intensity of ROS and GSH in bovine oocytes after in vitro maturation in the presence of nobiletin. Data are the mean ± SEM. Values with different superscript letters differ significantly ($p < 0.05$). Scale bar 50 µm.

Based on these results and to verify the effects of nobiletin on in vitro maturation and oxidative stress we analyzed gene expression in oocytes and their CCs. Only the experimental groups that showed better qualitative parameters in the previous experiments (Nob25and Nob50) were used in comparison with both control groups (Control and CDMSO). Supplementation of IVM medium with nobiletin, irrespective of the concentration, induced the upregulation of *MAPK1* and *BMP15* (developmental-related transcripts) and downregulation of *SOD2* and *CYP51A1* (oxidative stress transcripts) in oocytes after IVM when compared with control groups ($p < 0.05$). No significant differences were observed for the remaining transcripts studied (*BCL2*, *GAPDH*, *GDF9*) (Figure 4A). In CCs, nobiletin produced changes in the expression levels of genes related to quality and development (Figure 4B). *BMP15* (development) and *GJA1* (cell junctions) transcripts were upregulated ($p < 0.05$), while the expression of the oxidative stress (*SOD2*, *CYP51A1*) and apoptosis (*BCL2*) genes were downregulated in nobiletin groups compared to controls ($p < 0.05$). No significant differences were observed for the remaining transcripts studied (*ABCB1*, *CDH1*, *CLIC1*, *FOS*, *GAPDH*, *GDF9*, *IGF2R*, and *MAPK1*).

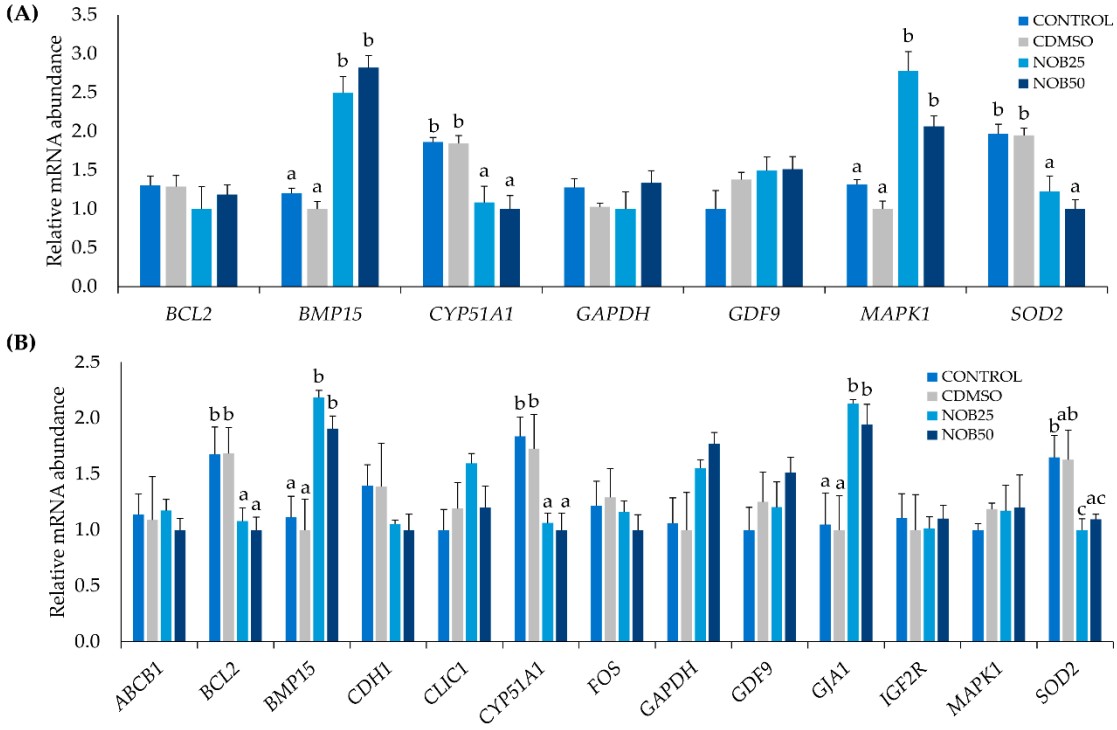

Figure 4. Relative mRNA transcript abundance (normalized against that of the endogenous control H2A histone family member Z (*H2AFZ*) gene and actin beta (*ACTB*)). (**A**) Bovine oocytes after in vitro maturation in the presence of nobiletin. (**B**) Bovine cumulus cells (CCs) after in vitro maturation in the presence of nobiletin. ATP-binding cassette subfamily B member 1 (*ABCB1*), BCL2- apoptosis regulator (*BCL2*), Bone morphogenetic protein 15 (*BMP15*), Cadherin 1 (*CDH1*), Chloride intracellular channel 1 (*CLIC1*), Cytochrome P450, family 51, subfamily A, polypeptide 1 (*CYP51A1*), Fos Proto-oncogene, AP-1 transcription factor subunit (*FOS*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Growth differentiation factor 9 (*GDF9*), Gap junction protein alpha 1 (*GJA1*), Insulin like growth factor 2 receptor (*IGF2R*), Mitogen-activated protein kinase 1 (*MAPK*), Superoxide Dismutase 2, Mitochondrial (former MnSOD) (*SOD2*). Data are the mean ± SEM. Different letters above columns indicate significant differences in gene expression among the experimental groups ($p < 0.05$).

2.2. Nobiletin Increases Estradiol (E_2) and Progesterone (P_4) Production by Cumulus Cells

After IVM, a significant increase in E_2 production by CCs was found in maturation medium supplemented with Nob25 (368.6 ± 27.3 pg/mL) and Nob50 (421.0 ± 28.2 pg/mL) compared with the rest

of the groups (Control: 233.2 ± 16.9 pg/mL; CDMSO: 212.4 ± 11.8 pg/mL; Nob10: 216.2 ± 20.0 pg/mL; and Nob100: 250.2 ± 24.4 pg/mL ($p < 0.05$; Figure 5A). Likewise, a significant increase in P_4 production by CCs in media after maturation was detected within Nob25 (19.7 ± 0.3 ng/mL) and Nob50 (20.2 ± 0.2 ng/mL) groups compared with the remaining groups ($p < 0.05$; Figure 5B).

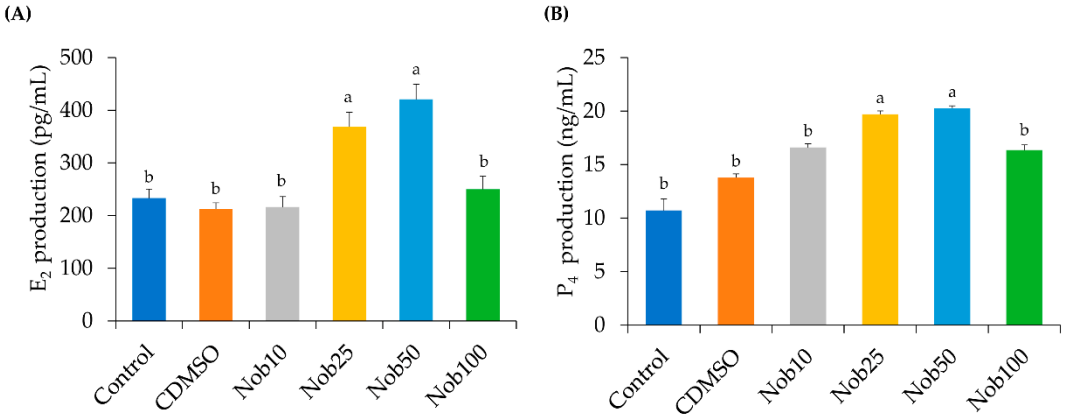

Figure 5. Steroidogenic production of cumulus cells (CCs) after in vitro maturation using different concentrations of nobiletin. (**A**) Steroidogenic production of Estradiol (E_2). (**B**) Steroidogenic production of Progesterone (P_4). Bars represent mean concentrations produced by CCs under each different experimental condition. Data are the mean ± SEM. Values with different superscript letters differ significantly ($p < 0.05$).

2.3. Nobiletin Increases Embryo Development and Quality

Embryonic development was assessed after IVM in the presence of nobiletin (Table 2). Cleavage rate and cumulative blastocyst yield at Day 7 and 8 were higher ($p < 0.05$) for Nob25 and Nob50 compared to all other groups. Based on these results, and for blastocysts quality evaluation only the Nob25 and Nob50 groups with both control groups (Control and CDMSO) were used for gene expression analysis.

Table 2. Effect of nobiletin on in vitro maturation of bovine oocytes and subsequent embryonic development.

Groups	Total No. Presumptive Zygotes in Culture	Cleavage Rate	Blastocyst Yield	
			Day 7	Day 8
		n (%)	*n* (%)	*n* (%)
Control	359	267 (74.2 ± 0.4)^b	76 (21.1 ± 0.4)^b	92 (25.8 ± 0.5)^b
CDMSO	378	278 (73.6 ± 0.5)^b	78 (20.9 ± 0.4)^b	98 (26.1 ± 0.7)^b
Nob10	397	300 (75.6 ±0.3)^b	75 (18.9 ± 0.4)^b	90 (23.1 ± 0.7)^b
Nob25	372	335 (89.9 ± 0.4)^a	90 (24.4 ± 0.5)^a	119 (32.2 ± 0.8)^a
Nob50	336	307 (91.3 ± 0.3)^a	86 (25.7 ± 0.6)^a	117 (35.3 ± 0.8)^a
Nob100	414	306 (74.0 ± 0.6)^b	76 (18.9 ± 0.9)^b	100 (24.5 ± 1.0)^b

n: number of oocytes assigned per group. Control: blastocysts cultured in the presence of SOF and 5% FCS; CDMSO: blastocysts cultured in the presence of SOF + 5% FCS supplemented with 0.01% DMSO; Nob10, Nob25, Nob50, Nob100 oocytes cultured in presence of SOF + 5% FCS supplemented with 10, 25, 50 and 100 µM nobiletin, respectively. Data are the mean ± SEM. Within columns, values with different superscript letters differ significantly ($p < 0.05$).

The expression of *MAPK1* was upregulated, while *CLIC1* was downregulated in blastocysts produced after oocyte maturation with nobiletin supplementation, irrespective of the concentration, compared with blastocysts from control groups ($p < 0.05$). The expression of *CYP51A1* was upregulated in blastocysts from the Nob50 group compared to blastocysts from control groups ($p < 0.05$).

No significant differences were observed for the remaining transcripts studied (*ABCB1*, *BCL2*, *BMP7*, *GAPDH*, *GDF9*, *IGF2R*, and *SOD2*) (Figure 6).

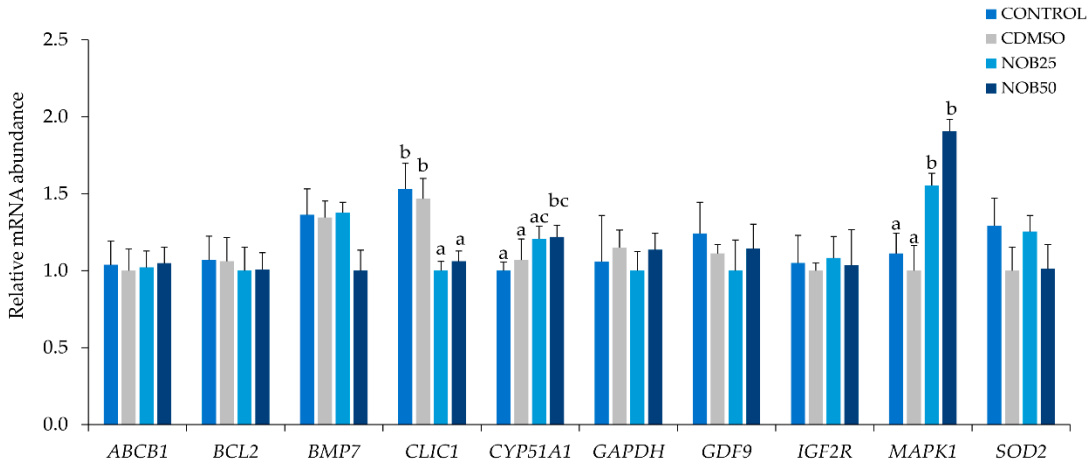

Figure 6. Relative mRNA transcript abundance (normalized against that of the endogenous control H2A histone family member Z (*H2AFZ*) gene and actin beta (*ACTB*)) of blastocysts D7 developed from oocytes matured in the presence of nobiletin. Data are the mean ± SEM. Different letters above columns indicate significant differences in gene expression among the experimental groups ($p < 0.05$).

3. Discussion

Nobiletin, a class of polymethoxylated flavone, has a broad range of biological effects including cell cycle regulation, reduction of apoptosis and antioxidation. To our knowledge, the present study is the first to investigate the effects of nobiletin supplementation in IVM on bovine oocyte quality and their developmental competence. We found that nobiletin while increases steroidogenesis of CCs, it also improves oocyte nuclear and cytoplasmic maturation (mitochondrial activity and CG migration) and decreases oocyte intracellular ROS and GSH levels, reflected to differentially expressed genes related to maturation, metabolism, cell communication, apoptosis and oxidative stress. Furthermore, nobiletin in IVM improves oocyte developmental competence and the quality of produced blastocyst in terms of the expression of genes linked to metabolism, development and oxidative stress.

Cumulus cells play an important role during oocyte growth and maturation, among them supply nutrients [14] and to mediate the effects of hormones during oocyte maturation [15]. Mingoti et al. [16] demonstrated that CCs of bovine COCs can secrete E_2 and P_4 in maturation media, and Endo et al. [17] and Sakaguchi et al. [18], demonstrated that exogenous and endogenous E_2 by granulosa cells directly supports the in vitro development of bovine COCs. In the present study, supplementation with 25 and 50 μM nobiletin in maturation medium increase in E_2 and P_4 production by CCs. This is in line with a study by Horigame et al. [19] that demonstrated that nobiletin enhanced testosterone production in cultures of Leydig cells via cAMP/CREB signaling. Therefore, our results indicated that nobiletin might act directly or synergistically with other hormones during oocyte maturation to alter the CCs steroidogenesis in vitro and that the increase of P_4 and E_2 production, without any steroid hormone supplementation, plays a positive role in oocyte nuclear and cytoplasmic maturation.

Nuclear maturation was improved by nobiletin supplementation to the IVM medium. This is in line with other studies using different antioxidants, such as resveratrol, astaxanthin or melatonin supplementation in bovine oocyte maturation in vitro [8,20]. However other studies in farm animals using a broad spectrum of antioxidants did not show an effect on the nuclear maturation rate, such as in pigs [5] and cattle [6]. These results suggest that different effects of antioxidants on nuclear maturation could be related to their capacity to activate the mitogen-activated protein kinase 1 (MAPK) pathway since in mammals, MAPK is responsible for meiotic progression [21], and in bovine oocytes, the two main isoforms (ERK1/2) of MAPK are activated near the time of germinal vesicle breakdown

(GVBD) [22]. One of the biological effects of nobiletin is the activation of MAPK activity shown in different cell types [10]. Hence, a more plausible explanation for an increase in M-II following nobiletin supplementation could be through P_4 and stimulation of MAPK1/ERK2, which plays a fundamental role in the regulation of microtubule organization, spindle assembly, chromosome distribution and meiosis resumption [23]. A similar function was demonstrated for resveratrol with improved meiosis resumption by enhancing the expression of Mos/MEK1/p42 MAPK cascade genes [8]. Based on the above, it could be hypothesized that nobiletin regulates the secretion of androgens in the CCs, and improved meiosis resumption by activation of MAPK; however, more experiments are necessary to corroborate this hypothesis.

Next, we observed that supplementation of nobiletin during in vitro maturation improved also cytoplasmic maturation. The migration of CG to the cortical region of the oocyte, as well as mitochondrial distribution and their activity, are suitable indicators to analyze cytoplasmic maturation [24,25]. Hosoe and Shioya [26] and Hoodbhoy et al. [27] demonstrated that proteins released by the CG are also necessary for preimplantation embryo development. We demonstrated that the addition of 25 or 50 μM nobiletin to IVM medium significantly increased peripheral distribution of CG, suggesting that nobiletin at these concentrations could act promoting a better organization of microfilaments and therefore, improving CG migration. A similar effect of CG migration was described with sodium nitroprusside for bovine oocytes in vitro maturation [28].

Mitochondria play an important role since they are a key component of the metabolic machinery responsible for the supply of energy that is consumed during the maturation process [29] and are also the main generator of free radicals in mammals [30]. The movement of mitochondria to areas of high energy consumption is crucial for the oocyte and the embryo during critical periods of the cell cycle. For this reason, the mitochondrial cytoplasmic distribution pattern has been associated with the quality and developmental capacity of mammalian oocytes and embryos [31,32]. We demonstrated that the addition of 25 or 50 μM nobiletin to IVM medium significantly increased mitochondrial migration, giving rise to granular aggregations throughout the cytoplasm in the oocyte after IVM. This pattern of distribution is similar to that described for bovine oocytes in other studies, which demonstrated that mitochondrial reorganization is necessary for cytoplasmic maturation, rearrangement of the cytoskeleton and developmental capacity after IVF [31,33,34]. Another important function of mitochondria is to synthesize adenosine triphosphate (ATP) through β-oxidation and this process involves the electron transport chain [4]. However, electrons may be lost during this process and could be bond to O_2, resulting in the production of ROS that decreases the developmental competence of the oocyte [35]. Thus, our results of increased oocyte mitochondrial activity could be related to the cytoprotective effects of nobiletin and its intrinsic ROS-scavenging property.

Under normal conditions, cells maintain their ROS levels in equilibrium [6], while during IVM, the cells may suffer disturbances in redox equilibrium having deleterious effects on development [3,36]. However, studies demonstrated that the addition of antioxidants into the culture medium reduces the harmful effects of ROS during IVM and offers a way of protecting the oocyte and subsequent embryo [5,6]. In the oocyte, the main ROS scavenger system is GSH that uses a reducing power provided by oxidative metabolism [3]. Our results show that 25 or 50 μM nobiletin supplementation in IVM medium reduced the intracellular ROS levels, which is in agreement with the use of other flavonoids such as resveratrol [8], quercetin and taxifolin [37] and other class of antioxidants like vitamin C [6]. Regarding nobiletin, studies in cell cultures demonstrated its ability to significantly decrease ROS generation [13] but to date, there are no studies available on its effects in oocytes and embryos. Nobiletin has a beneficial effect on cell protection [10], and like other antioxidants, this effect could be produced due to its hydrophobic nature, which allows it to incorporate into the membrane [37], inhibiting ROS attack and decreasing lipid peroxidation. Therefore, the positive effect observed in the present study could be attributed to this property; nonetheless, further studies are necessary to understand the mechanism of its antioxidant effects in oocytes.

In cytoplasmic maturation, GSH is considered a biochemical marker for oocyte quality, and plays an important role in maintaining redox homeostasis, hence protecting the embryo from oxidative damage before genomic activation [7]. Our results showed decreased levels of GSH in 25 or 50 μM nobiletin supplemented groups, opposed to other studies reporting either a reduction in ROS levels associated with an increase in GSH levels [6,8] or no increase in GSH levels in bovine oocytes [3]. A reasonable explanation for this could be due to the highest mitochondrial activity found in the oocytes from Nob25 and Nob50 groups. It is widely known that increased mitochondrial activity leads to an increase in the exchange of electrons in the inner mitochondrial membrane, which is considered one of the main sources of ROS production [38]. Despite a high mitochondrial activity, the intracellular ROS levels in the mature oocytes from the Nob25 and Nob50 groups were lower than those observed in the oocytes from the control groups, suggesting that GSH was consumed to avoid the harmful effects of the high levels of ROS. This explanation has been proposed before by Rocha-Frigoni et al. [3] for cysteine and cysteamine antioxidant activity during bovine oocyte IVM and by Qu et al. [39] for nobiletin reduction of ROS levels in response to cadmium-induced neuronal injury in rats.

Improvements in oocyte quality by 25 and 50 μM nobiletin supplementation during in vitro maturation were reflected by increased blastocyst development rates on Day 7 and 8. These results are in line with other studies which evaluated other flavonoids like resveratrol [8], or antioxidants such as cysteamine [6], vitamin C [40], lycopene [41], and carnitine [42] in the IVM medium. Furthermore, flavonoids or antioxidants in the IVM showed an interaction with the expression of certain qualitatively related genes to the development of mature oocytes and/or the production of blastocysts.

To test if the effects of nobiletin during IVM were related to gene expression changes, we analyzed the expression of candidate genes for oxidative stress, embryo development, and quality. Superoxide dismutase 2 (*SOD2*), an indicator of oxidative stress [42] was downregulated in oocytes and CCs obtained from Nob25 and Nob50 groups compared with controls, whereas in blastocysts it was not altered. This is in accordance with the findings of Gülcin [43], who showed that superoxide plays an important role in the neutralization of ROS, so a reduction in ROS formation requires less *SOD2* to neutralize free radicals. On the other hand, Chloride intracellular channel 1 (*CLIC1*) is considered as a sensor of cell oxidation [44,45] and is involved in ROS production [45]. Our results showed that *CLIC1* was downregulated in blastocysts obtained from Nob25 and Nob50 groups compared with the controls, both also with increased embryo yield, which agree with earlier studies showing that *CLIC1* expression accompanied by low accumulation of ROS improves embryo development [46]. These findings together with the low intracellular ROS and GSH levels in the oocytes matured with nobiletin supplementation indicate an improvement of their antioxidant activity and consequently an enhanced quality of the produced blastocysts.

Cytochrome P450 family 51 subfamily A polypeptide 1 (*CYP51A1*), Bone morphogenic protein 15 (*BMP15*), Mitogen-activated protein kinase 1 (*MAPK1*), Gap junction alpha-1 protein (*GJA1*) and *BCL2*-apoptosis regulator (*BCL2*), are genes considered quality biomarkers of in vitro matured oocytes [47,48]. *CYP51A1* participates in the regulation of cholesterol biosynthesis [49] and it has been demonstrated that biosynthesis of cholesterol is one example of metabolic cooperation between granulosa cells and oocytes [50]. Furthermore, the upregulation of the enzyme coded by *CYP51A1* is a result of negative feedback reflecting lowered cholesterol availability, which is implicated in the lower quality of oocytes [49]. Therefore, downregulation of *CYP51A1* mRNA expression observed in oocytes and their CCs matured with nobiletin supplementation could be an indicator of good quality. In contrast, 50 μM nobiletin supplementation in IVM upregulated the expression of *CYP51A1* in blastocysts. This is in line with the results of nobiletin supplementation in liver cell culture (HepG2), showing upregulation of CYP1 (Cytochrome P450s family) and improved cholesterol synthesis due to full methoxylation in the A-ring of nobiletin chemical structure [10,51]. Hence, nobiletin could act differently depending on the cell type, probably due to the bioactivity or its chemical structure, which causes that *CYP51A1* might be down or upregulated to control cholesterol availability, however, more in deep studies are necessary to corroborate this information.

In mammals, *BMP15* is known to be involved in oocyte maturation and cholesterol biosynthesis, being specifically expressed in oocytes and acting on CCs, improving oocyte competence, and early embryo development in cattle [52,53]. Several studies reported an increase in *BMP15* transcript during maturation in buffalo [54] and dog [55] oocytes, which are consistent with our findings of an increase in *BMP15* expression in oocytes and their CCs matured with nobiletin supplementation in IVM, related with their improved developmental competence.

The MAPK family plays an important role in bovine oocyte maturation by inducing GVBD [56]. Likewise, *MAPK1* mRNA plays a key role in oocyte maturation by acting on granulosa and CCs in various species including cattle [22] and dogs [55]. Our results demonstrated that *MAPK1* mRNA expression in oocytes and embryos was upregulated, suggesting that nobiletin could act on cell cycle regulation as reported by Yoshimizu et al. [57] and Morley et al. [58] in other types of cells. On the other hand, *GJA1*, also known as connexin 43 (Cx43), is a component of gap junctions expressed in CCs and a major mediator of cell-to-cell communication via gap junctions, and a proliferation regulator [59]. Recently, it was shown that CCs of bovine oocytes with higher developmental competence express higher *GJA1* [60]. These findings are in agreement with our results demonstrating higher *GJA1* expression in the CCs from oocytes matured with nobiletin. Taken together, these results suggest that nobiletin modifies the expression of key genes for oocyte cytoplasmic development and maturation, improving their developmental competence and increasing embryo yield.

Moreover, we observed that during IVM, nobiletin decreased the expression of *BCL2* in CCs. The downregulation of *BCL2* expression is associated with a protective effect and has been reported to have a critical role in CCs by acting as a regulator of apoptosis [61]. Studies in cattle showed that lycopene (antioxidant) supplementation during in vitro maturation, increases expression of *BCL2* exerting a pro-apoptotic effect [41]. Studies that used nobiletin on human cancer cell lines (gastric, hepatic, and breast) shown that nobiletin induced apoptotic cell death by reducing the expression of *BCL2* [10,12,58]. However, the molecular mechanisms whereby nobiletin induces apoptosis among different carcinogenic cells remain poorly understood. Therefore, it is to be assumed that nobiletin has different actions for healthy and unhealthy cells.

In conclusion, a concentration 25 or 50 μM nobiletin offers a novel alternative for counteracting the effects of the increase in the production of ROS during IVM and subsequent embryo development in cattle. In matured oocytes and their cumulus cells, nobiletin modifies the expression of genes involved in maturation (*BMP15* and *MAPK1*), metabolism (*CYP51A1*), communication (*GJA1*), apoptosis (*BCL2*) and oxidative stress (*SOD2* and *CLIC1*), which was reflected in the increased nuclear and cytoplasmic maturation (mitochondrial activity and CG migration) and CCs steroidogenesis, decreased intracellular ROS and GSH levels, as well as enhanced embryo development and quality. These benefits of nobiletin can be attributed to its bioactivity, chemical structure, and antioxidant properties, and might be a tool to overcome ROS disorders in bovine IVP embryos and to improve ART in mammals.

4. Materials and Methods

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA).

4.1. Oocyte Collection and In Vitro Maturation

Immature cumulus-oocyte complexes (COCs) were obtained by aspirating follicles (2–8 mm diameter) from the ovaries of mature heifers (i.e., at least one corpus luteum or remained scars from previous ovulations in one or both ovaries) collected at local slaughterhouses. A total of 3758 class 1 and 2 COCs (homogeneous cytoplasm and intact CCs) were matured in groups of 50 COCs per well for 24 h, at 38.5 °C under an atmosphere of 5% CO_2 in air, with maximum humidity [2] in 500 μL of maturation medium, TCM-199 with 10% (*v/v*) fetal calf serum (FCS) and 10 ng/mL epidermal growth factor (Control, $n = 595$); supplemented either with 10, 25, 50, and 100 μM nobiletin (MedChemExpress, MCE, Sollentuna, Sweden); (Nob10, $n = 645$; Nob25, $n = 630$; Nob50, $n = 603$; and Nob100, $n = 672$,

respectively) or dimethyl sulfoxide (DMSO control (CDMSO), 0.01% DMSO vehicle for nobiletin dilution, $n = 613$). The concentration of nobiletin was based on the findings of other studies in which this polymethoxylated flavonoid was used in vivo in zebrafish and chick embryos and in vitro in human umbilical vein endothelial cells, showing an anti-angiogenic activity at concentrations between 30 and 100 µM [10,39,62].

After 24 h of IVM, a representative number of matured COCs under different conditions were employed to evaluate: nuclear maturation, cortical granules migration (CG), mitochondria (Mt) distribution patterns and mitochondrial activity, levels of ROS and GSH and mRNA abundance of selected genes (oocytes and their CCs). The remaining oocytes were processed for in vitro fertilization and culture to assess their developmental competence. To analyze the mRNA abundance of selected genes, four pools of 10 matured COCs were collected from each treatment, and CCs were physically separated from oocytes by gentle pipetting in phosphate-buffered saline (PBS). Oocytes, in pools of 10 per treatment group, were washed in PBS, snap-frozen in liquid N_2 (LN_2), and stored at −80 °C until mRNA extraction. Their corresponding CCs were also washed in PBS, centrifuged at 10,000 g, and then snap-frozen in LN_2 and stored at −80 °C until mRNA extraction.

To measure the steroidogenic production of COCs after IVM, media from all groups were collected and stored at −20 °C until analysis.

4.2. Cortical Granules (CG) Distribution Patterns

Visualization of CG distribution was performed according to Arias-Álvarez et al. [63], with minor modifications. Briefly, in vitro matured COCs from each treatment were first suspended in 100 µL of PBS without calcium or magnesium supplemented with 0.1% polyvinylpyrrolidone (PVP) and their CCs were removed by gentle pipetting. Next, oocytes were treated with 0.5% (*w/v*) pronase to digest the zona pellucida. Zona-free oocytes were washed in PBS + 0.1% PVP three times and fixed in 4% (*w/v*) buffered neutral paraformaldehyde (PF) solution (pH 7.2–7.4) for 30 min at room temperature and then treated with permeabilization solution (0.02% *v/v* Triton X-100 in PBS + 1% Bovine Serum Albumin (BSA) for 10 min). The oocytes were then treated for 30 min with blocking solution (7.5% *w/v* BSA in PBS) and incubated in 100 µg/mL FITC-labeled Lens culinaris (LCA-FITC, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature in a dark chamber. Following, oocytes were treated for 30 min with Hoechst 33342 (10 µg/mL) to evaluate nuclear maturation. After staining, oocytes were washed in PBS + 0.1% PVP, mounted in 3.8 µL of mounting medium (50% *v/v* PBS, 50% *v/v* glycerol, 0.5 µg/mL Hoechst) between a coverslip and a glass slide and sealed with nail polish. Slides were examined using a laser-scanning confocal microscope (Leica TCS SP2; Leica Microsystems GmbH, Wetzlar, Germany) equipped with an argon laser excited at 488 nm and whose detection spectrum is 515 nm.

As a measure of cytoplasmic maturation, CG distribution was analyzed (Control: $n = 58$; CDMSO: $n = 66$; Nob10: $n = 72$; Nob25: $n = 70$; Nob50: $n = 78$; Nob100: $n = 70$) and classified as: non-migrated (CGs distributed throughout the cytoplasm); partially migrated (CGs dispersed and partly clustered throughout the cortical area); and migrated (small CG arranged at the periphery or adjacent to the plasma membrane) [26,29]. Simultaneously, oocytes were evaluated for nuclear maturation.

4.3. Mitochondrial Distribution Patterns and Quantification of Mitochondrial Activity

Briefly, in vitro matured COCs from each treatment were first suspended in 100 µL PBS + 0.1% PVP and their CCs were removed by gentle pipetting. Next, oocytes were equilibrated for 15 min in maturation medium and then placed in four-well culture plates containing 500 µL of 400 nM MitoTracker DeepRed (Molecular Probes Inc., Eugene, OR, USA) per well. The plates were incubated at 38.5 °C, 5% CO_2 in the dark, and humidified atmosphere for 30 min. The stained oocytes were washed twice in PBS + 0.1% PVP and fixed in 4% PF for 30 min at room temperature. Following, oocytes were treated for 30 min with Hoechst 33342 (10 µg/mL) for evaluating nuclear maturation. After that, oocytes were washed in PBS + 0.1% PVP, mounted in 3.8 µL of mounting medium between

a coverslip and a glass slide and sealed with nail polish. Slides were examined using a laser-scanning confocal microscope (Leica TCS SP2) equipped with an argon laser excited at 644 nm with a detection spectrum of 625–665 nm. The format, laser, gain, and offset were kept constant for every sample. Serial sections of 5 µm were made for each oocyte and a maximum projection was accomplished for each.

Mitochondrial patterns and mitochondrial activity were analyzed in matured oocytes from Control: $n = 59$; CDMSO: $n = 56$; Nob10: $n = 61$; Nob25: $n = 76$; Nob50: $n = 71$; Nob100: $n = 74$. The distribution was classified as: non-migrated (when mitochondria were homogeneously distributed throughout the cytoplasm); partially migrated (mitochondria were heterogeneously distributed throughout the cytoplasm) and migrated (mitochondria were distributed with granular aggregations) [1,29,33,38]. For the assessment of mitochondrial activity, the fluorescence signal intensity (pixels) was quantified. Images obtained were evaluated using the ImageJ program (NIH, ImageJ version 1.52k software (http://rsbweb.nih.gov/ij/), using the freehand selection tool. Fluorescence intensity in each oocyte was determined using the following formula: Relative fluorescence = integrated density (IntDen) − (area of selected oocyte x mean fluorescence of background readings). Fluorescence intensities are expressed in arbitrary units (a.u.) [3,46]. Simultaneously, these oocytes were evaluated for nuclear maturation.

4.4. Assessment of Oocyte Nuclear Maturation

Matured oocytes from all treatments stained for CG distribution and mitochondrial distribution and activity were also stained with Hoechst 33342 solution (10 µg/mL of PBS) for nuclear chromosomal and polar body evaluation (Control: $n = 117$; CDMSO: $n = 122$; Nob10: $n = 133$; Nob25: $n = 146$; Nob50: $n = 149$; Nob100: $n = 144$). Oocytes were classified as follows: immature oocytes comprising the stages of germinal vesicle (GV, nucleus well defined), germinal vesicle breakdown (GVBD, chromosome condensation), metaphase I (MI, first metaphasic plate visible); and matured oocytes comprising the stage of metaphase-II (M-II, represented by the presence of the first polar body and/or the second metaphasic plate). Nuclear maturation was assessed under an epifluorescence microscope (Nikon 141731, Tokyo, Japan) equipped with a fluorescent lamp (Nikon HB-10104AF) and UV-1 filter. Oocytes in M-II were considered as matured.

4.5. Levels of Reactive Oxygen Species (ROS) and Glutathione (GSH)

For evaluation of ROS and GSH, in vitro matured COCs from each treatment (Control: $n = 54$; CDMSO: $n = 48$; Nob10: $n = 50$; Nob25: $n = 47$; Nob50: $n = 53$; Nob100: $n = 49$), were first suspended in 100 µL PBS + 0.1% PVP and their CCs were removed by gentle pipetting, then were incubated in four-well plates containing 500 µL of 10 µM of CellROX Deep Red Reagent (Invitrogen, Eugene, OR, USA) for ROS and 20 µM of CellTracker Fluorescent (Molecular Probes, Eugene, OR, USA) for GSH per well, at 38.5 °C, 5% CO_2 in a dark and humidified atmosphere for 30 min. After staining, oocytes were washed twice with PBS+ 0.1% PVP, mounted in 3.8 µL of mounting medium between a coverslip and a glass slide, sealed with nail polish, and were imaged immediately using an epifluorescence microscope (Nikon 141731). Fluorescence emitted from the oocytes was captured using B-2E/C (ROS) and UV-2A (GSH) filters for ten seconds after exposure to UV light. The digital images were processed and analyzed using ImageJ. The relative ROS and GSH fluorescence intensity in each oocyte were assessed as described for the mitochondrial activity (Section 4.3.).

4.6. Steroidogenic Production of Estradiol and Progesterone by CCs

Progesterone (P_4) concentration was measured in spent maturation media by solid-phase radioimmunoassay method (RIA) using the methods as described by Santiago-Moreno et al. [64]. Aliquots of 100 µl were used in duplicate, then each of the samples was measured in the liquid Scintillation Counter (Tri-Carb®2100TR) including the measurement of the standard curve. The intra-assay coefficient of variation was 11% and assay sensitivity was 0.4 ng/mL. Estradiol (E_2) concentrations in spent maturation media were measured by a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding specific kit (DEH3355

DEMEDITEC Diagnostics GmbH, Kiel, Germany) according to the manufacturer´s instructions. Intra-assay coefficients of variation were 6%. Results are expressed as average E_2 (pg/mL) and P_4 (ng/mL) concentrations produced by 50 COCs after the IVM period using 3 replicates.

4.7. Sperm Preparation and In Vitro Fertilization (IVF)

IVF was performed as described previously [65]. Briefly frozen semen straws (0.25 mL) from an Asturian Valley bull previously tested for IVF were thawed at 37 °C in a water bath for 1 min and centrifuged for 10 min at 280 g through a gradient of 1 mL of 40% and 1 mL of 80% Bovipure (Nidacon Laboratories AB, Göthenborg, Sweden), according to the manufacturer´s instructions. The sperm pellet was isolated and washed in 3 mL of Boviwash (Nidacon Laboratories AB, Göthenborg, Sweden) by centrifugation at 280 *g* for 5 min. The pellet was re-suspended in the remaining 300 µL of Boviwash. The final concentration of spermatozoa was adjusted to 1×10^6 spermatozoa/mL. Gametes were co-incubated for 18–22 h in 500 µL fertilization media (Tyrode's medium) with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, and 6 mg/mL fatty acid-free bovine serum albumin (BSA) supplemented with 10 mg/mL heparin sodium salt (Calbiochem) in four-well cell culture plates in groups of 50 COCs per well under an atmosphere of 5% CO_2 in the air, with maximum humidity at 38.5 °C.

4.8. In Vitro Culture of Presumptive Zygotes

At 18–22 h post-insemination (hpi), presumptive zygotes from each experimental group (Control: n = 359; CDMSO: n = 378; Nob10: n = 397; Nob25: n = 372; Nob50: n = 336; Nob100: n = 414) were denuded of CCs by vortexing for 3 min and then cultured in groups of 25 in 25 µL droplets of culture medium (synthetic oviductal fluid (SOF) [66]); with 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 µL/mL BME amino acids, 10 µL/mL minimum essential medium (MEM) amino acids and 1 µg/mL phenol red supplemented with 5% FCS under mineral oil at 38.5 °C under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 with maximum humidity. Cleavage rate was recorded at day 2 (48 hpi) and cumulative blastocyst yield was determined on Days 7 and 8 pi. Pools of ten Day 7 expanding blastocysts from each treatment group were washed in PBS, snap-frozen in LN_2, and stored at −80 °C until mRNA extraction.

4.9. Gene Expression Analysis

Gene expression analysis was performed using four pools of 10 oocytes, and their corresponding CCs and four pools of 10 Day 7 expanded blastocysts per treatment group. All samples were washed in PBS, snap-frozen in LN_2, and stored at −80 °C until mRNA extraction analyses.

Poly(A) RNA was extracted using the Dynabeads mRNA Direct Extraction Kit (Ambion; Thermo Fisher Scientific Inc., Oslo, Norway) with minor modifications [67]. Immediately after poly(A) RNA extraction, reverse transcription (RT) was performed using an Moloney murine leukemia virus (MMLV) Reverse Transcriptase 1st-Strand cDNA Synthesis Kit according to the manufacturer's instructions (Epicentre Technologies Corp., Madison, WI, USA). Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure, allowing Poly(T) random primers and Oligo dT annealing, and the RT mix was then completed by adding 0.375 mM dNTPs (Biotools, Madrid, Spain), 6.25 U RNAsin RNAse inhibitor (Promega, Madison, WI, USA), MMLV HP RT 10x reaction buffer, 5 mM DTT and 5 U MMLV high-performance reverse transcriptase. Samples were incubated at 25 °C for 10 min, and then at 37 °C for 60 min, to allow the RT of RNA, and finally at 85 °C for 5 min to denature the enzyme. All mRNA transcripts were quantified in duplicate using a Rotorgene 6000 Real-Time Cycler (Corbett Research, Sydney, Australia). RT–quantitative polymerase chain reaction (qPCR) was performed by adding a 2 µL aliquot of each cDNA sample (~60 ng μL^{-1}) to the PCR mix (GoTaq qPCR Master Mix, Promega, Madrid, Spain) containing specific primers to amplify the genes of interest. Primer sequences are provided in Supplementary Table S1. The selection of genes to be evaluated in oocytes, CCs and blastocysts was carried out considering that they are representative of

key processes, i.e., communication (*GJA1*), oxidative stress (*SOD2, GAPDH*), metabolism (*CYP51A1),* quality (*BCL2, GDF9, IGF2R*) and development (*BMP15, CLIC1, ABCB1, BMP7, MAPK1, CDH1*) as previously described by [40,46]. All primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries when possible. For quantification, RT-qPCR was performed as described previously [68]. The PCR conditions were tested to achieve efficiencies close to 1. Relative expression levels were quantified by the comparative cycle threshold (CT) method [69]. Values were normalized using two housekeeping (HK) genes: *H2AFZ* and *ACTB.* Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the mean CT value of the two housekeeping genes from the CT value of the gene of interest in the same sample. The calculation of ΔΔCT involved using the highest treatment ΔCT value (i.e., the treatment with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold-changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

4.10. Statistical Analysis

All statistical tests were performed using the software package SigmaStat (Systat Software Inc., San Jose, CA, USA). Nuclear maturation, CG and mitochondrial distribution patterns, mitochondrial activity, ROS, and GSH measurements, steroidogenic production of estradiol and progesterone, cleavage and blastocysts rates and relative mRNA abundance were normally distributed with homogeneous variance, so one-way analysis of variance (ANOVA), followed by Tukey´s test, was performed to evaluate the significance of differences between groups. Values were considered significantly different at $p < 0.05$. Unless otherwise indicated, data are presented as the mean ± SEM.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/15/5340/s1. Figure S1: Quantification of mitochondria activity in bovine oocytes after in vitro maturation in the presence of nobiletin. Control (n = 59): oocytes cultured in the presence of SOF and 5% FCS; CDMSO (n = 56): oocytes cultured in the presence of SOF + 5% FCS supplemented with 0.01% DMSO; Nob10 (n = 61), Nob25 (n = 76), Nob50 (n = 71), Nob100 (n = 74) oocytes cultured in presence of SOF + 5% FCS supplemented with 10, 25, 50 and 100 μM nobiletin, respectively, Table S1: Summary of primer sequences used for RT-qPCR in oocytes, CCs and blastocysts after in vitro maturation in the presence of nobiletin.

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References

1. Zhang, T.; Fan, X.; Li, R.; Zhang, C.; Zhang, J. Effects of pre-incubation with C-type natriuretic peptide on nuclear maturation, mitochondrial behavior, and developmental competence of sheep oocytes. *Biochem. Biophys. Res. Commun.* **2018**, *497*, 200–206. [CrossRef]
2. Rizos, D.; Ward, F.; Duffy, P.; Boland, M.P.; Lonergan, P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* **2002**, *61*, 234–248. [CrossRef]
3. Rocha-Frigoni, N.A.; Leão, B.C.D.S.; Dall'Acqua, P.C.; Mingoti, G. Improving the cytoplasmic maturation of bovine oocytes matured in vitro with intracellular and/or extracellular antioxidants is not associated with increased rates of embryo development. *Theriogenology* **2016**, *86*, 1897–1905. [CrossRef] [PubMed]
4. Ambrogi, M.; Dall'Acqua, P.C.; Rocha-Frigoni, N.; Leão, B.C.D.S.; Mingoti, G. Transporting bovine oocytes in a medium supplemented with different macromolecules and antioxidants: Effects on nuclear and cytoplasmic maturation and embryonic development in vitro. *Reprod. Domest. Anim.* **2017**, *52*, 409–421. [CrossRef] [PubMed]
5. Kere, M.; Siriboon, C.; Lo, N.-W.; Nguyen, N.T.; Ju, J.-C. Ascorbic Acid Improves the Developmental Competence of Porcine Oocytes After Parthenogenetic Activation and Somatic Cell Nuclear Transplantation. *J. Reprod. Dev.* **2012**, *59*, 78–84. [CrossRef] [PubMed]
6. Sovernigo, T.; Adona, P.R.; Monzani, P.; Guemra, S.; Barros, F.; Lopes, F.; Leal, C. Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production. *Reprod. Domest. Anim.* **2017**, *52*, 561–569. [CrossRef] [PubMed]
7. De Matos, D.; Furnus, C. The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development: Effect of β-mercaptoethanol, cysteine and cystine. *Theriogenology* **2000**, *53*, 761–771. [CrossRef]
8. Wang, F.; Tian, X.; Zhang, L.; He, C.; Ji, P.; Li, Y.; Tan, D.; Liu, G. Beneficial effect of resveratrol on bovine oocyte maturation and subsequent embryonic development after in vitro fertilization. *Fertil. Steril.* **2014**, *101*, 577.e1–586.e1. [CrossRef]
9. Kimura, O.; Ohta, C.; Koga, N.; Haraguchi, K.; Kato, Y.; Endo, T. Carrier-mediated uptake of nobiletin, a citrus polymethoxyflavonoid, in human intestinal Caco-2 cells. *Food Chem.* **2014**, *154*, 145–150. [CrossRef]
10. Huang, H.; Li, L.; Shi, W.; Liu, H.; Yang, J.; Yuan, X.; Wu, L. The Multifunctional Effects of Nobiletin and Its MetabolitesIn VivoandIn Vitro. *Evid.-Based Complement. Altern. Med.* **2016**, *2016*, 1–14. [CrossRef]
11. Liu, B.; Huang, J.; Zhang, B. Nobiletin protects against murine l -arginine-induced acute pancreatitis in association with downregulating p38MAPK and AKT. *Biomed. Pharmacother.* **2016**, *81*, 104–110. [CrossRef] [PubMed]
12. Chen, C.; Ono, M.; Takeshima, M.; Nakano, S. Antiproliferative and apoptosis-inducing activity of nobiletin against three subtypes of human breast cancer cell lines. *Anticancer. Res.* **2014**, *34*, 1785–1792. [PubMed]
13. Choi, S.-Y.; Hwang, J.-H.; Ko, H.-C.; Park, J.-G.; Kim, S.J. Nobiletin from citrus fruit peel inhibits the DNA-binding activity of NF-κB and ROS production in LPS-activated RAW 264.7 cells. *J. Ethnopharmacol.* **2007**, *113*, 149–155. [CrossRef] [PubMed]
14. Eppig, J.J. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. *Dev. Boil.* **1982**, *89*, 268–272. [CrossRef]
15. Zuelke, K.A.; Brackett, B.G. Luteinizing hormone-enhanced in vitro maturation of bovine oocytes with and without protein supplementation. *Boil. Reprod.* **1990**, *43*, 784–787. [CrossRef]
16. Mingoti, G.; Garcia, J.; Rosa-E-Silva, A. Steroidogenesis in cumulus cells of bovine cumulus–oocyte-complexes matured in vitro with BSA and different concentrations of steroids. *Anim. Reprod. Sci.* **2002**, *69*, 175–186. [CrossRef]
17. Endo, M.; Kawahara-Miki, R.; Cao, F.; Kimura, K.; Kuwayama, T.; Monji, Y.; Iwata, H. Estradiol supports in vitro development of bovine early antral follicles. *Reproduction* **2012**, *145*, 85–96. [CrossRef]
18. Sakaguchi, K.; Tanida, T.; Abdel-Ghani, M.A.; Kanno, C.; Yanagawa, Y.; Katagiri, S.; Nagano, M. Relationship between the antral follicle count in bovine ovaries from a local abattoir and steroidogenesis of granulosa cells cultured as oocyte-cumulus-granulosa complexes. *J. Reprod. Dev.* **2018**, *64*, 503–510. [CrossRef]

19. Horigome, S.; Maeda, M.; Ho, H.-J.; Shirakawa, H.; Komai, M. Effect of Kaempferia parviflora extract and its polymethoxyflavonoid components on testosterone production in mouse testis-derived tumour cells. *J. Funct. Food.* **2016**, *26*, 529–538. [CrossRef]
20. Pang, Y.-W.; Zhao, S.; Sun, Y.; Jiang, X.; Hao, H.; Du, W.; Zhu, H. Protective effects of melatonin on the in vitro developmental competence of bovine oocytes. *Anim. Sci. J.* **2017**, *89*, 648–660. [CrossRef]
21. Fan, H.-Y.; Sun, Q.-Y. Involvement of Mitogen-Activated Protein Kinase Cascade During Oocyte Maturation and Fertilization in Mammals1. *Boil. Reprod.* **2004**, *70*, 535–547. [CrossRef] [PubMed]
22. Quetglas, M.D.; Adona, P.; De Bem, T.; Pires, P.; Leal, C.L.V. Effect of Cyclin-dependent Kinase (CDK) Inhibition on Expression, Localization and Activity of Maturation Promoting Factor (MPF) and Mitogen Activated Protein Kinase (MAPK) in Bovine Oocytes. *Reprod. Domest. Anim.* **2009**, *45*, 1074–1081. [CrossRef]
23. Gordo, A.C.; He, C.L.; Smith, S.; Fissore, R. Mitogen activated protein kinase plays a significant role in metaphase II arrest, spindle morphology, and maintenance of maturation promoting factor activity in bovine oocytes. *Mol. Reprod. Dev.* **2001**, *59*, 106–114. [CrossRef] [PubMed]
24. El-Raey, M.; Geshi, M.; Somfai, T.; Kaneda, M.; Hirako, M.; Abdel-Ghaffar, A.E.; Sosa, G.A.; El-Roos, M.E.A.; Nagai, T. Evidence of melatonin synthesis in the cumulus oocyte complexes and its role in enhancing oocyte maturation in vitro in cattle. *Mol. Reprod. Dev.* **2011**, *78*, 250–262. [CrossRef] [PubMed]
25. Mo, X.; Wu, G.; Yuan, D.; Jia, B.; Liu, C.; Zhu, S.; Hou, Y. Leukemia inhibitory factor enhances bovine oocyte maturation and early embryo development. *Mol. Reprod. Dev.* **2014**, *81*, 608–618. [CrossRef]
26. Hosoe, M.; Shioya, Y. Distribution of cortical granules in bovine oocytes classified by cumulus complex. *Zygote* **1997**, *5*, 371–376. [CrossRef]
27. Hoodbhoy, T.; Dandekar, P.; Calarco, P.; Talbot, P. p62/p56 are cortical granule proteins that contribute to formation of the cortical granule envelope and play a role in mammalian preimplantation development. *Mol. Reprod. Dev.* **2001**, *59*, 78–89. [CrossRef]
28. Viana, K.; Caldas-Bussiere, M.C.; Matta, S.; Faes, M.; De Carvalho, C.P.; Quirino, C. Effect of sodium nitroprusside, a nitric oxide donor, on the in vitro maturation of bovine oocytes. *Anim. Reprod. Sci.* **2007**, *102*, 217–227. [CrossRef]
29. Ferreira, E.; Vireque, A.A.; Adona, P.; Meirelles, F.; Ferriani, R.; Navarro, P. Cytoplasmic maturation of bovine oocytes: Structural and biochemical modifications and acquisition of developmental competence. *Theriogenology* **2009**, *71*, 836–848. [CrossRef]
30. Brand, M.D.; Orr, A.; Perevoshchikova, I.; Quinlan, C.L. The role of mitochondrial function and cellular bioenergetics in ageing and disease. *Br. J. Dermatol.* **2013**, *169*, 1–8. [CrossRef]
31. Tarazona, A.; Rodriguez, J.; Restrepo, L.; Olivera-Ángel, M. Mitochondrial Activity, Distribution and Segregation in Bovine Oocytes and in Embryos Produced in Vitro. *Reprod. Domest. Anim.* **2006**, *41*, 5–11. [CrossRef] [PubMed]
32. Egerszegi, I.; Alm, H.; Rátky, J.; Heleil, B.; Brüssow, K.-P.; Torner, H. Meiotic progression, mitochondrial features and fertilisation characteristics of porcine oocytes with different G6PDH activities. *Reprod. Fertil. Dev.* **2010**, *22*, 830–838. [CrossRef] [PubMed]
33. Lima, F.; Bezerra, F.; Souza, G.; Matos, M.; Hurk, R.V.D.; Silva, J.R.V. Influence of interleukin 1 beta and tumour necrosis factor alpha on the in vitro growth, maturation and mitochondrial distribution of bovine oocytes from small antral follicles. *Zygote* **2018**, *26*, 381–387. [CrossRef] [PubMed]
34. Zhao, X.-M.; Wang, N.; Hao, H.-S.; Li, C.-Y.; Zhao, Y.-H.; Yan, C.-L.; Wang, H.-Y.; Du, W.; Wang, D.; Liu, Y.; et al. Melatonin improves the fertilization capacity and developmental ability of bovine oocytes by regulating cytoplasmic maturation events. *J. Pineal Res.* **2017**, *64*, e12445. [CrossRef]
35. Barja, G. Mitochondrial Oxygen Consumption and Reactive Oxygen Species Production are Independently Modulated: Implications for Aging Studies. *Rejuvenation Res.* **2007**, *10*, 215–224. [CrossRef]
36. Pamplona, R.; Costantini, D. Molecular and structural antioxidant defenses against oxidative stress in animals. *Am. J. Physiol. Integr. Comp. Physiol.* **2011**, *301*, R843–R863. [CrossRef]
37. Kang, J.-T.; Moon, J.H.; Choi, J.-Y.; Park, S.J.; Kim, S.J.; Saadeldin, I.M.; Lee, B.C. Effect of Antioxidant Flavonoids (Quercetin and Taxifolin) on In vitro Maturation of Porcine Oocytes. *Asian-Australas. J. Anim. Sci.* **2016**, *29*, 352–358. [CrossRef]
38. Zhao, X.-M.; Du, W.-H.; Wang, D.; Hao, H.-S.; Liu, Y.; Qin, T.; Zhu, H.-B. Recovery of mitochondrial function and endogenous antioxidant systems in vitrified bovine oocytes during extended in vitro culture. *Mol. Reprod. Dev.* **2011**, *78*, 942–950. [CrossRef]

39. Qu, Y.; Liu, Y.; Chen, L.; Zhu, Y.; Xiao, X.; Wang, D.; Zhu, Y. Nobiletin prevents cadmium-induced neuronal apoptosis by inhibiting reactive oxygen species and modulating JNK/ERK1/2 and Akt/mTOR networks in rats. *Neurol. Res.* **2018**, *40*, 211–220. [CrossRef]
40. Torres, V.; Hamdi, M.; Maillo, V.; Urrego, R.; Echeverri, J.J.; López-Herrera, A.; Gutierrez-Adan, A.; Rizos, D.; Sánchez-Calabuig, M.J. Ascorbic acid-cyclodextrin complex alters the expression of genes associated with lipid metabolism in bovine in vitro produced embryos. *Reprod. Domest. Anim.* **2019**, *54*, 55–62. [CrossRef]
41. Chowdhury, M.; Choi, B.-H.; Khan, I.; Lee, K.-L.; Mesalam, A.; Song, S.-H.; Xu, L.; Joo, M.-D.; Afrin, F.; Kong, I.-K. Supplementation of lycopene in maturation media improves bovine embryo quality in vitro. *Theriogenology* **2017**, *103*, 173–184. [CrossRef] [PubMed]
42. Mishra, A.; Reddy, I.; Gupta, P.; Mondal, S. l-carnitine Mediated Reduction in Oxidative Stress and Alteration in Transcript Level of Antioxidant Enzymes in Sheep Embryos Produced In Vitro. *Reprod. Domest. Anim.* **2016**, *51*, 311–321. [CrossRef] [PubMed]
43. Gulçin, I. Antioxidant and antiradical activities of l-carnitine. *Life Sci.* **2006**, *78*, 803–811. [CrossRef]
44. Goodchild, S.C.; Howell, M.W.; Cordina, N.M.; Littler, D.; Breit, S.N.; Curmi, P.M.G.; Brown, L.J. Oxidation promotes insertion of the CLIC1 chloride intracellular channel into the membrane. *Eur. Biophys. J.* **2009**, *39*, 129–138. [CrossRef] [PubMed]
45. Averaimo, S.; Milton, R.H.; Duchen, M.R.; Mazzanti, M. Chloride intracellular channel 1 (CLIC1): Sensor and effector during oxidative stress. *FEBS Lett.* **2010**, *584*, 2076–2084. [CrossRef]
46. Hamdi, M.; Lopera-Vasquez, R.; Maillo, V.; Calabuig, M.-J.S.; Núnez, C.; Gutierrez-Adan, A.; Rizos, D. Bovine oviductal and uterine fluid support in vitro embryo development. *Reprod. Fertil. Dev.* **2018**, *30*, 935. [CrossRef]
47. Salhab, M.; Dhorne-Pollet, S.; Auclair, S.; Guyader-Joly, C.; Brisard, D.; Dalbiès-Tran, R.; Dupont, J.; Ponsart, C.; Mermillod, P.; Uzbekova, S. In vitro maturation of oocytes alters gene expression and signaling pathways in bovine cumulus cells. *Mol. Reprod. Dev.* **2013**, *80*, 166–182. [CrossRef]
48. Li, S.-H.; Lin, M.-H.; Hwu, Y.-M.; Lu, C.-H.; Yeh, L.-Y.; Chen, Y.-J.; Lee, R.K.-K. Correlation of cumulus gene expression of GJA1, PRSS35, PTX3, and SERPINE2 with oocyte maturation, fertilization, and embryo development. *Reprod. Boil. Endocrinol.* **2015**, *13*, 93. [CrossRef]
49. Nakamura, T.; Iwase, A.; Bayasula, B.; Nagatomo, Y.; Kondo, M.; Nakahara, T.; Takikawa, S.; Goto, M.; Kotani, T.; Kiyono, T.; et al. CYP51A1 Induced by Growth Differentiation Factor 9 and Follicle-Stimulating Hormone in Granulosa Cells Is a Possible Predictor for Unfertilization. *Reprod. Sci.* **2014**, *22*, 377–384. [CrossRef]
50. Su, Y.-Q.; Sugiura, K.; Eppig, J.J. Mouse Oocyte Control of Granulosa Cell Development and Function: Paracrine Regulation of Cumulus Cell Metabolism. *Semin. Reprod. Med.* **2009**, *27*, 32–42. [CrossRef]
51. Noguchi, S.; Atsumi, H.; Iwao, Y.; Kan, T.; Itai, S. Nobiletin: A citrus flavonoid displaying potent physiological activity. *Acta Crystallogr. Sect. C Struct. Chem.* **2016**, *72*, 124–127. [CrossRef] [PubMed]
52. Hussein, T.S.; Sutton-McDowall, M.L.; Gilchrist, R.B.; Thompson, J.G. Temporal effects of exogenous oocyte-secreted factors on bovine oocyte developmental competence during IVM. *Reprod. Fertil. Dev.* **2011**, *23*, 576–584. [CrossRef] [PubMed]
53. Caixeta, E.S.; Sutton-McDowall, M.L.; Gilchrist, R.B.; Thompson, J.G.; Price, C.A.; Machado, M.F.; Lima, P.F.; Buratini, J. Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus–oocyte complexes. *Reproduction* **2013**, *146*, 27–35. [CrossRef] [PubMed]
54. Kathirvel, M.; Eswari, S.; Kumanan, V. Differential expression dynamics of Growth differentiation factor9 (GDF9) and Bone morphogenetic factor15 (BMP15) mRNA transcripts during in vitro maturation of buffalo (Bubalus bubalis) cumulus–oocyte complexes. *SpringerPlus* **2013**, *2*, 206. [CrossRef]
55. Lee, S.H.; Oh, H.J.; Kim, M.J.; A Kim, G.; Bin Choi, Y.; Jo, Y.K.; Setyawan, E.M.N.; Lee, B.C. Oocyte maturation-related gene expression in the canine oviduct, cumulus cells, and oocytes and effect of co-culture with oviduct cells on in vitro maturation of oocytes. *J. Assist. Reprod. Genet.* **2017**, *34*, 929–938. [CrossRef]
56. Inoue, M. Mitogen-activated protein kinase translocates into the germinal vesicle and induces germinal vesicle breakdown in porcine oocytes. *Boil. Reprod.* **1998**, *58*, 130–136. [CrossRef]

57. Yoshimizu, N.; Otani, Y.; Saikawa, Y.; Kubota, T.; Yoshida, M.; Furukawa, T.; Kumai, K.; Kameyama, K.; Fujii, M.; Yano, M.; et al. Anti-tumour effects of nobiletin, a citrus flavonoid, on gastric cancer include: Antiproliferative effects, induction of apoptosis and cell cycle deregulation. *Aliment. Pharmacol. Ther.* **2004**, *20*, 95–101. [CrossRef]
58. Morley, K.L.; Ferguson, P.J.; Koropatnick, J. Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. *Cancer Lett.* **2007**, *251*, 168–178. [CrossRef]
59. Brązert, M.; Kranc, W.; Nawrocki, M.J.; Sujka-Kordowska, P.; Konwerska, A.; Jankowski, M.; Kocherova, I.; Celichowski, P.; Jeseta, M.; Ożegowska, K.; et al. New markers for regulation of transcription and macromolecule metabolic process in porcine oocytes during in vitro maturation. *Mol. Med. Rep.* **2020**, *21*, 1537–1551. [CrossRef]
60. Read, C.; Willhelm, G.; Dyce, P.W. Connexin 43 coupling in bovine cumulus cells, during the follicular growth phase, and its relationship to in vitro embryo outcomes. *Mol. Reprod. Dev.* **2018**, *85*, 579–589. [CrossRef]
61. Boelhauve, M.; Sinowatz, F.; Wolf, E.; Paula-Lopes, F.F. Maturation of Bovine Oocytes in the Presence of Leptin Improves Development and Reduces Apoptosis of In Vitro-Produced Blastocysts1. *Boil. Reprod.* **2005**, *73*, 737–744. [CrossRef]
62. Lam, K.H.; Alex, D.; Lam, I.K.; Tsui, S.K.; Yang, Z.F.; Lee, S.M. Nobiletin, a polymethoxylated flavonoid from citrus, shows anti-angiogenic activity in a zebrafish in vivo model and HUVEC in vitro model. *J. Cell. Biochem.* **2011**, *112*, 3313–3321. [CrossRef]
63. Alvarez, M.A.; García-García, R.M.; Rebollar, P.; Nicodemus, N.; Revuelta, L.; Millan, P.; Lorenzo, P.L. Effects of a lignin-rich fibre diet on productive, reproductive and endocrine parameters in nulliparous rabbit does. *Livest. Sci.* **2009**, *123*, 107–115. [CrossRef]
64. Santiago-Moreno, J.; Gómez-Brunet, A.; Gonzalez-Bulnes, A.; Toledano, A.; Malpaux, B.; López-Sebastián, A. Differences in reproductive pattern between wild and domestic rams are not associated with inter-specific annual variations in plasma prolactin and melatonin concentrations. *Domest. Anim. Endocrinol.* **2005**, *28*, 416–429. [CrossRef]
65. Lopera-Vasquez, R.; Hamdi, M.; Maillo, V.; Lloreda, V.; Coy, P.; Gutierrez-Adan, A.; Bermejo-Álvarez, P.; Rizos, D. Effect of bovine oviductal fluid on development and quality of bovine embryos produced in vitro. *Reprod. Fertil. Dev.* **2017**, *29*, 621. [CrossRef]
66. Holm, P.; Booth, P.; Schmidt, M.; Greve, T.; Callesen, H. High bovine blastocyst development in a static in vitro production system using sofaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology* **1999**, *52*, 683–700. [CrossRef]
67. Bermejo-Alvarez, P.; Rizos, D.; Rath, D.; Lonergan, P.; Gutierrez-Adan, A. Epigenetic differences between male and female bovine blastocysts produced in vitro. *Physiol. Genom.* **2008**, *32*, 264–272. [CrossRef]
68. Bermejo-Álvarez, P.; Rizos, D.; Rath, D.; Lonergan, P.; Gutierrez-Adan, A. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 3394–3399. [CrossRef]
69. Schmittgen, T.D.; Livak, K.J. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* **2008**, *3*, 1101–1108. [CrossRef]

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Nobiletin enhances the development and quality of bovine embryos in vitro during two key periods of embryonic genome activation

Karina Cañón-Beltrán[1,2,6], Yulia N. Cajas[1,6], Serafín Peréz-Cerezales[1], Claudia L. V. Leal[1,3], Ekaitz Agirregoitia[4], Alfonso Gutierrez-Adán[1], Encina M. González[5] & Dimitrios Rizos[1]✉

In vitro culture can alter the development and quality of bovine embryos. Therefore, we aimed to evaluate whether nobiletin supplementation during EGA improves embryonic development and blastocyst quality and if it affects PI3K/AKT signaling pathway. In vitro zygotes were cultured in SOF + 5% FCS (Control) or supplemented with 5, 10 or 25 µM nobiletin (Nob5, Nob10, Nob25) or with 0.03% dimethyl-sulfoxide (C_{DMSO}) during minor (2 to 8-cell stage; MN_{EGA}) or major (8 to 16-cell stage; MJ_{EGA}) EGA phase. Blastocyst yield on Day 8 was higher in Nob5 (42.7 ± 1.0%) and Nob10 (44.4 ± 1.3%) for MN_{EGA} phase and in Nob10 (61.0 ± 0.8%) for MJ_{EGA} phase compared to other groups. Mitochondrial activity was higher and lipid content was reduced in blastocysts produced with nobiletin, irrespective of EGA phase. The mRNA abundance of *CDK2*, *H3-3B*, *H3-3A*, *GPX1*, *NFE2L2* and *PPARα* transcripts was increased in 8-cells, 16-cells and blastocysts from nobiletin groups. Immunofluorescence analysis revealed immunoreactive proteins for p-AKT forms (Thr308 and Ser473) in bovine blastocysts produced with nobiletin. In conclusion, nobiletin supplementation during EGA has a positive effect on preimplantation bovine embryonic development in vitro and corroborates on the quality improvement of the produced blastocysts which could be modulated by the activation of AKT signaling pathway.

In vitro culture (IVC) of bovine embryos is one of the most important processes in the development of assisted reproductive techniques due to the fact that postfertilization culture conditions can dramatically alter the quality of the resulting blastocysts[1,2]. In vitro, gametes and embryos are exposed to spatial and temporal unnatural conditions, whose scope is not completely known[3]. Although many improvements have been made, in vitro culture systems are still not as efficient as in vivo embryo production[4]. In cattle, the proportion of embryos reaching the blastocyst stage is around 30–40%[5] and are often compromised in quality and competence manifested by a darker morphology[1] or altered gene expression patterns[6] when compared to their *in vivo* counterparts. The factors that most influence the quality of the embryos are the conditions after fertilization; which include physicochemical (temperature, osmolality, and pH), oxidative (antioxidant balance), and energetic (production, utilization, and storage) stresses[6].

Under in vitro conditions, the dynamics of embryo development and the kinetics of cleavage are related to the subsequent developmental stages: the faster-cleaved embryos have a higher chance to develop to the blastocysts stage[7]. Therefore, the morphological and metabolic changes that occur during the first 4 days of preimplantation development of the bovine embryo are the most important; besides, during this same period the embryonic genome activation (EGA) occurs[2,8]. At the start of early embryogenesis, all mRNAs and proteins controlling development are of maternal origin, and as development progresses, these reserves gradually degrade while

[1]Department of Animal Reproduction, National Institute for Agriculture and Food Research and Technology (INIA), 28040 Madrid, Spain. [2]Departamento de Ciencias Biológicas, Universidad Técnica Particular de Loja, Loja 110107, Ecuador. [3]Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga 13635-900, Brazil. [4]Department of Physiology, Faculty of Medicine and Nursing, Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), 48940 Bizkaia, Spain. [5]Department of Anatomy and Embryology, Veterinary Faculty, Complutense University of Madrid (UCM), 28040 Madrid, Spain. [6]These authors contributed equally: Karina Cañón-Beltrán and Yulia N. Cajas. ✉email: drizos@inia.es

embryonic transcripts are synthesized; this process is called maternal-to-embryonic transition and involves EGA[9]. The EGA occurs in distinct waves, which are species-specific. Bovine preimplantation embryo development is characterized by two distinct phases: (i) minor EGA (MN_{EGA}) (2-cell to 8-cell stages) where zygotes and early embryos are transcriptionally and translationally active; (ii) major EGA (MJ_{EGA}) (8-cell to 16-cells stages) which includes a gradual degradation of mRNA molecules of maternal origin, together with a change in the protein synthesis, and these events are key factors for successful embryonic development and differentiation[2,8]. EGA is a prerequisite for correct compaction, that leads to an increase in intercellular adhesion mediated by adherent junctions and embryonic polarization[10], as well as the formation of the blastocyst, with its trophectoderm (TE), and the inner cell mass (ICM)[11].

In recent years, the role of different signaling pathways in preimplantation development has been analyzed, suggesting the existence of a complex network of signals that control and are responsible for cell division, differentiation, cytoskeleton rearrangements, cell proliferation and apoptosis[11,12]. One of the most important signal transduction pathways that regulate cell survival is PI3K/AKT. PI3K/AKT pathway consists of several molecules, including kinases, phosphatases, and transcription factors that are fundamental in processes such as migration, metabolism, and cell cycle progression[13,14]. During embryonic development, PI3K/AKT regulates cell survival and its inhibition can cause a significant delay in blastocyst hatching[12]. In this context, the quality of the embryos produced in vitro depends not only on the proper functioning of the signaling pathways but also on the post-fertilization culture environment.

To improve the blastocysts rates and quality, several studies have probed the addition of different types of natural antioxidants to the IVC medium, such as vitamin C[15] or crocetin[16]. These compounds improved embryonic quality in terms of increase in blastocysts rates and embryo cell number, as well as reduction in reactive oxygen species (ROS) levels and apoptotic cells in embryos. In recent years, nobiletin a class of polymethoxylated flavone identified from the citrus peel (chemically known as 5,6,7,8,3′,4′ hexamethoxyflavone), has drawn increasing attention since it is easily absorbed across the cytoplasmic membranes due to its structure and lipophilic nature[17]. In addition, it has been reported that nobiletin has a broad spectrum of biological activities, that include antioxidative functions and cell cycle regulation[17]. We observed that supplementation of *in vitro* maturation (IVM) medium with nobiletin counteracts the effects of the increase in ROS production during IVM, improves oocyte nuclear and cytoplasmic maturation, and subsequent embryo development and quality in bovine[18]. Other studies using cultured cell lines have demonstrated that nobiletin can modulate signaling cascades, including PI3K/AKT signaling pathway[17,19]. Nevertheless, the mechanism of specific action by which nobiletin modulates this signaling pathway is not fully understood, and, to our knowledge, there is no evidence of any developmental effect of nobiletin supplementation during post-fertilization embryo culture in vitro. Thus, in this study, we aimed to evaluate whether supplementation of nobiletin to the in vitro culture medium during the two EGA phases improves embryonic development and blastocyst quality and if its action is related to the PI3K/AKT signaling pathway. The parameters evaluated in blastocysts were, (i) lipid accumulation, (ii) mitochondrial activity, (iii) quantitative changes of key genes related to quality and development, (iv) immunolocalization of phosphorylated-AKT (p-AKT) and (v) level by western blot analysis for AKT and p-AKT (Thr308/Ser473).

Results

Nobiletin during MN_{EGA} or MJ_{EGA} enhances early embryo development in vitro. For all experimental groups, only embryos that reached the 8-cell stage at 54 h post-insemination (hpi) were selected for the study. As shown in Fig. 1a for MN_{EGA} phase, no differences were observed in cleavage rate at 54 hpi, which ranged from 82.3 ± 1.0 to 85.5 ± 0.5%. At 54 hpi, no differences were observed either in the proportion of embryos that reached the 8-cell stage, which ranged from 57.1 ± 1.4 to 60.4 ± 0.7%. Consequently, a similar proportion of embryos with a delayed development (< 8 cells), which ranged from 22.6 ± 0.9 to 26.6 ± 1.2%, was observed (Fig. 1b). Blastocyst yield at Day 7 and 8 (Fig. 1c) was significantly higher ($P < 0.001$) for Nob5 (39.7 ± 0.8 and 42.7 ± 1.0%, respectively) and Nob10 (41.0 ± 1.0 and 44.4 ± 1.3%), compared to Control (32.7 ± 0.7 and 34.6 ± 0.7%); C_{DMSO} (32.8 ± 0.5 and 34.9 ± 0.4%) and Nob25 (31.8 ± 1.7 and 34.6 ± 1.2%) (Supplementary Table S1).

During MJ_{EGA}, cleavage rate at 54 hpi was 86.6 ± 0.2% and the proportion of embryos that reached the 8-cell stage was 71.1 ± 0.4% while the proportion of embryos with a delayed development (< 8 cells) was 15.5 ± 0.3% (Fig. 1d). At 96 hpi a significantly ($P < 0.001$) higher proportion of embryos reached the 16-cell stage in Nob5 and Nob10 groups (70.1 ± 0.5% and 69.9 ± 0.4%, respectively) compared to Control (60.0 ± 0.4%), C_{DMSO} (60.7 ± 0.4%) and Nob25 (60.8 ± 0.8%) groups (Fig. 1e). As a consequence, a significantly lower proportion of embryos with a delayed development (<16 cells) was observed in Nob5 and Nob10 compared to the other groups (Nob5: 29.9 ± 0.5% and Nob10: 30.1 ± 0.4% vs Control: 40.0 ± 0.4%, C_{DMSO}: 39.3 ± 0.4% and Nob25: 39.2 ± 0.8%, $P < 0.001$). On Day 7 and 8, blastocyst yield was significantly higher ($P < 0.001$) for Nob10 (54.5 ± 1.1% and 61.0 ± 0.8%, respectively) compared to Control (38.4 ± 1.1% and 47.3 ± 1.4%), C_{DMSO} (35.8 ± 1.0% and 44.0 ± 1.1%), Nob5 (46.6 ± 0.8% and 52.5 ± 1.5%) and Nob25 (35.9 ± 1.5%–42.5 ± 1.3%) groups, while Nob5 was higher ($P < 0.001$) compared to Nob25 and both control groups (Fig. 1f) (Supplementary Table S2).

Nobiletin during MN_{EGA} or MJ_{EGA} increases the quality of in vitro produced blastocysts. Only the experimental groups that showed better blastocyst yield in the previous experiment (Nob5 and Nob10 during MN_{EGA} or MJ_{EGA}) were used for embryo quality evaluation in comparison with both control groups (Control and C_{DMSO}).

The mitochondrial activity was higher ($P < 0.001$) in blastocysts from Nob5 and Nob10 groups, from either MN_{EGA} or MJ_{EGA} phase, compared with both control groups (Fig. 2).

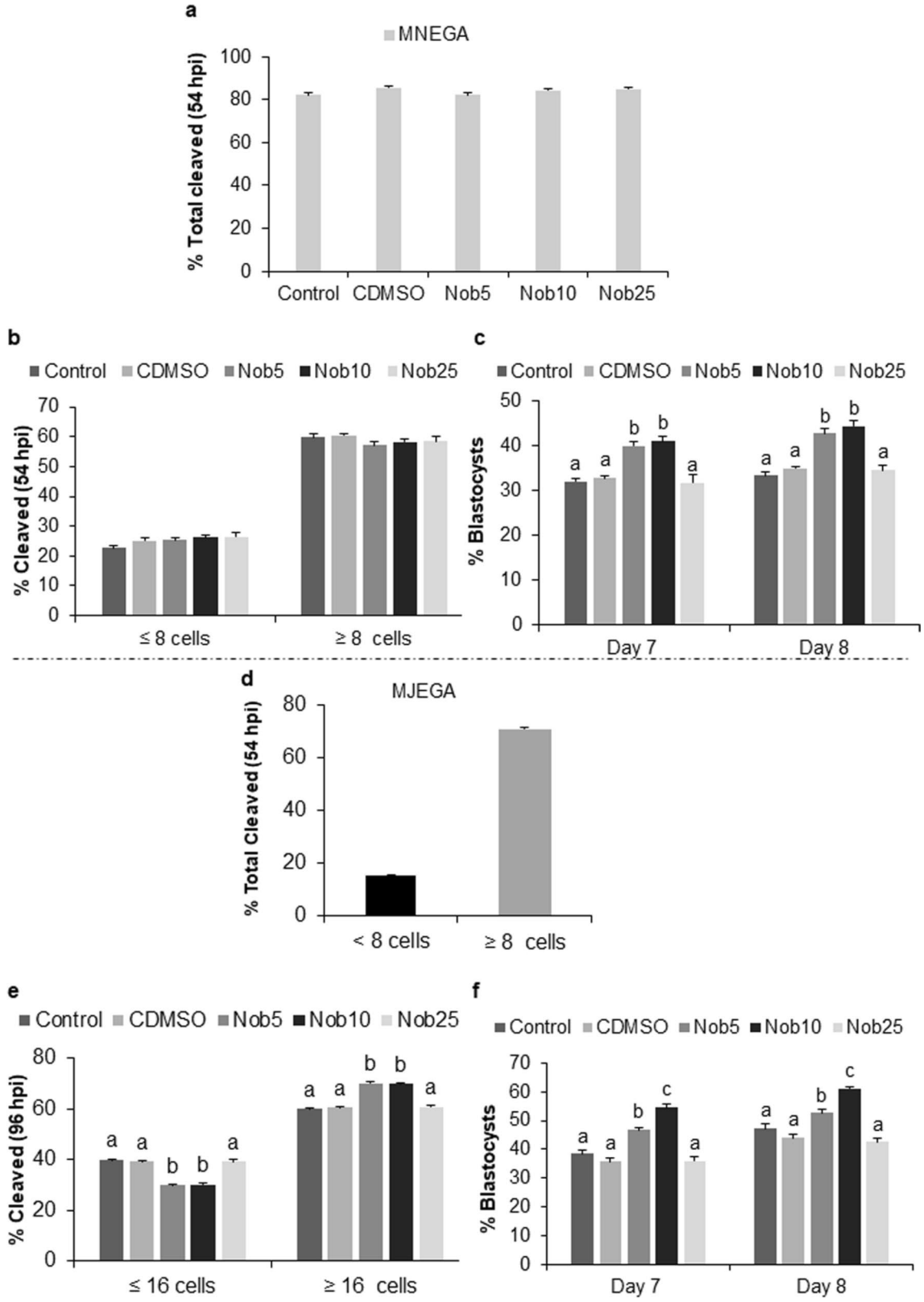

Figure 1. Nobiletin effect in embryonic development. Developmental rates of in vitro produced bovine embryos cultured during 21–54 h post-insemination (hpi) (MN_{EGA}: **a**–**c**) or during 54–96 hpi (MJ_{EGA}: **d**–**f**) with or without nobiletin. (**a**,**d**) Total cleavage rate at 54 hpi; (**b**,**e**) embryos ≥ or ≤ 8-cell stage at 54 hpi and ≥ or ≤ 16-cell stage at 96 hpi; (**c**,**f**) blastocyst rate on Days 7–8 pi (in vitro fertilization = Day 0), from embryos cultured in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5), 10 (Nob10) or 25 μM (Nob25) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}) during MN_{EGA} or MJ_{EGA} respectively. Results are expressed as mean ± s.e.m. Significant differences ($P < 0.001$) are indicated with different letters.

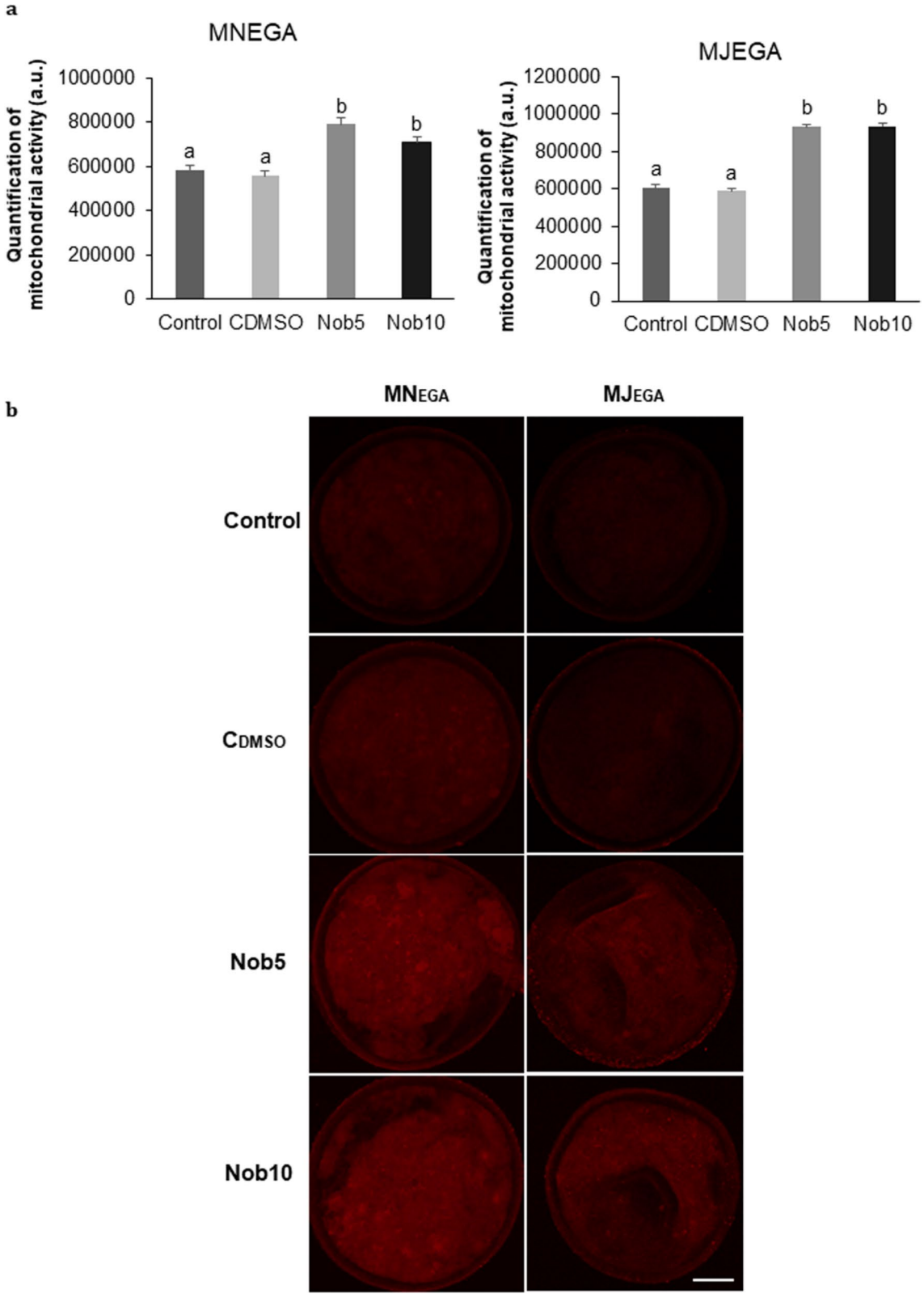

Figure 2. Nobiletin effect in blastocysts mitochondrial activity. (**a**) Quantification of mitochondrial fluorescence intensity in arbitrary units (a.u) in Day 7 blastocysts cultured in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5) or 10 μM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}) during 21–54 hpi (MN_{EGA}: presumptive zygote to 8-cell stage) or during 54–96 hpi (MJ_{EGA}: 8- to 16-cell stage). Data are the mean ± s.e.m. Significant differences ($P < 0.001$) are indicated with different letters. (**b**) Representative fluorescence images of mitochondrial activity in Day 7 blastocysts from all experimental groups (Control, Nob5 Nob10, C_{DMSO}) in both phases (MN_{EGA} or MJ_{EGA}). Images were captured on 63× objective. Scale bar 50 μm.

When analyzing the lipid content, we observed that the total area of lipid droplets in blastocysts resulting from treatments during MN_{EGA} or MJ_{EGA} was significantly reduced ($P < 0.001$) in Nob5 and Nob10 groups compared with the control groups (Fig. 3).

The total number of cells was greater ($P < 0.001$) in blastocysts from MN_{EGA} phase produces with 5 µM of nobiletin (137.3 ± 0.6) compared to all other groups (Control: 105.7 ± 0.7; C_{DMSO}: 106.4 ± 0.8; Nob10: 126.7 ± 0.8), while blastocysts from Nob10 group had more cells ($P < 0.001$) compared to control groups, but less ($P < 0.001$) when compared to Nob5. However, during MJ_{EGA} phase the total number of cells was higher in blastocysts from Nob5 and Nob10 groups (133.2 ± 0.9 and 134.2 ± 0.7, respectively) compared to control groups (Control: 104.9 ± 0.7 and C_{DMSO}: 104.6 ± 0.6) ($P < 0.001$) (Supplementary Table S3).

Gene expression in ≥ 8-cell embryos and blastocysts produced with nobiletin during MN_{EGA}. The mRNA abundance of *CDK2, H3-3B, H3-3A,* and *GPX1* was significantly increased in 8-cell stage embryos from Nob5 and Nob10 groups compared to both controls ($P < 0.05$) (Fig. 4a). The expression of *PPARα* and *GPX1* was significantly higher in blastocysts from Nob5 and Nob10 groups when compared with both controls ($P < 0.05$) (Fig. 4b). No differences were observed for the *PPARGC1A, PPARα, RPS6KB1,* and *NFE2L2* transcripts in 8-cell stage embryos and *PPARGC1A, RPS6KB1, CDK2, H3-3B, H3F3A,* and *NFE2L2* in blastocysts.

Gene expression in ≥ 16-cell embryos and blastocysts produced with nobiletin during MJ_{EGA}. The expression level of *CDK2, H3-3B* and *NFE2L2* transcripts was significantly increased in 16-cell stage embryos from Nob10 group compared to Nob5 and both control groups. While the expression of *GPX1* gene was higher in Nob5 and Nob10 compared to control groups ($P < 0.05$) (Fig. 5a). In blastocysts the expression of *PPARα* was significantly higher in Nob10 group compared to all other groups ($P < 0.05$), while *CDK2* and *GPX1* were upregulated in both nobiletin groups compared with controls ($P < 0.05$) (Fig. 5b). No significant differences were observed for *PPARGC1A, PPARα, RPS6KB1* and *H3-3A* in 16-cell stage embryos, and for *PPARGC1A, RPS6KB1, H3-3B, H3-3A* and *NFE2L2* in blastocysts.

Nobiletin during MN_{EGA} or MJ_{EGA} increases AKT phosphorylation in blastocysts produced in vitro. Immunofluorescence analysis revealed immunoreactive proteins for p-AKT in bovine blastocysts. In Day 7 blastocysts, AKT increased its phosphorylation levels when nobiletin was present in the culture medium (Nob5 and Nob10 groups) during MN_{EGA} or MJ_{EGA}. While p-AKT levels were weaker in blastocysts produced from control groups during MJ_{EGA} phase (Fig. 6).

Similarly, the western blot analysis showed that both p-AKT-Thr308 and p-AKT-Ser473 phosphorylation levels were significantly higher in blastocysts produced with nobiletin supplementation (Nob5 and Nob10) during MN_{EGA} phase when compared with control groups ($P < 0.05$) (Fig. 7a–c). A similar pattern was observed in response to nobiletin treatment during MJ_{EGA}, as p-AKT-Thr308 and p-AKT-Ser473 phosphorylation levels were significantly higher in blastocysts produced with Nob5 and Nob10 compared with control groups ($P < 0.05$) (Fig. 7d–f).

Discussion

Under in vivo conditions, cells have antioxidants levels in equilibrium and possess physiological mechanisms to hinder excessive free radical formation[20]. During in vitro culture this mechanism suffers disturbances, in which the redox balance is altered with an increase in the production of free radicals and, as a consequence, a decrease in embryo development[6]. Several studies, aiming to identify the most effective antioxidants to reduce the alteration of the redox balance and ROS levels during the in vitro production of embryos, have shown that the addition of quercetin, resveratrol, vitamin C or carnitine to the culture media have beneficial effects on early embryonic development[15,21]. To our knowledge, the present study is the first that investigates the antioxidant effects of nobiletin supplementation in the culture medium during the two main phases of EGA (MN_{EGA}: minor activation from 2- to 8-cell stage and MJ_{EGA}: major activation from 8- to 16-cell stage)[2,8] in bovine embryo developmental competence in vitro and quality of the produced blastocysts, as well as its possible interaction with the AKT signaling pathway.

Irrespective of concentration, addition of nobiletin to culture media during MN_{EGA} phase (21–54 hpi) did not affect cleavage rates at 54 hpi as well as the percentage of embryos reach the 8-cell stage but increased blastocyst production, whereas nobiletin supplementation in culture media during MJ_{EGA} phase (54–96 hpi) significantly increased the percentage of embryos that reach the16-cell stage and blastocyst production. Several studies have shown that during EGA the bovine embryo actively synthesize transcription factors and this process directly links to chromatin changes, protein allocation, nuclear reorganization and cell proliferation[8,22]. Since in our results developmental kinetics were stimulated with more 16-cell embryos by nobiletin during MJ_{EGA}, we could hypothesize that nobiletin activates early embryonic genes important for the proper genomic function of the embryo during major EGA. Although with our experimental design we cannot link this effect specifically with either of the two activation phases of the embryonic genome.

The evidence that nobiletin supplementation improves blastocyst production is in line with other studies showing increased embryo development in vitro when culture medium was supplemented with biological antioxidants similar to nobiletin[16,23,24]. Another effect of nobiletin was to induce a significant increase in mitochondrial activity and a lower content of lipid droplets in blastocysts from both EGA phases analyzed. Mitochondria play a central role in the generation of adenosine triphosphate (ATP), so, they are considered as energy control units necessary for cell division, pluripotency and differentiation[25]. Cagnone and Sirard[6], reported that in vivo, during the early cleavages, mitochondria and intracellular metabolism are quiescent. Nevertheless, in vitro, this metabolic quiescence is altered due to the presence of nutrients in excessive amounts that overstimulate mitochondria

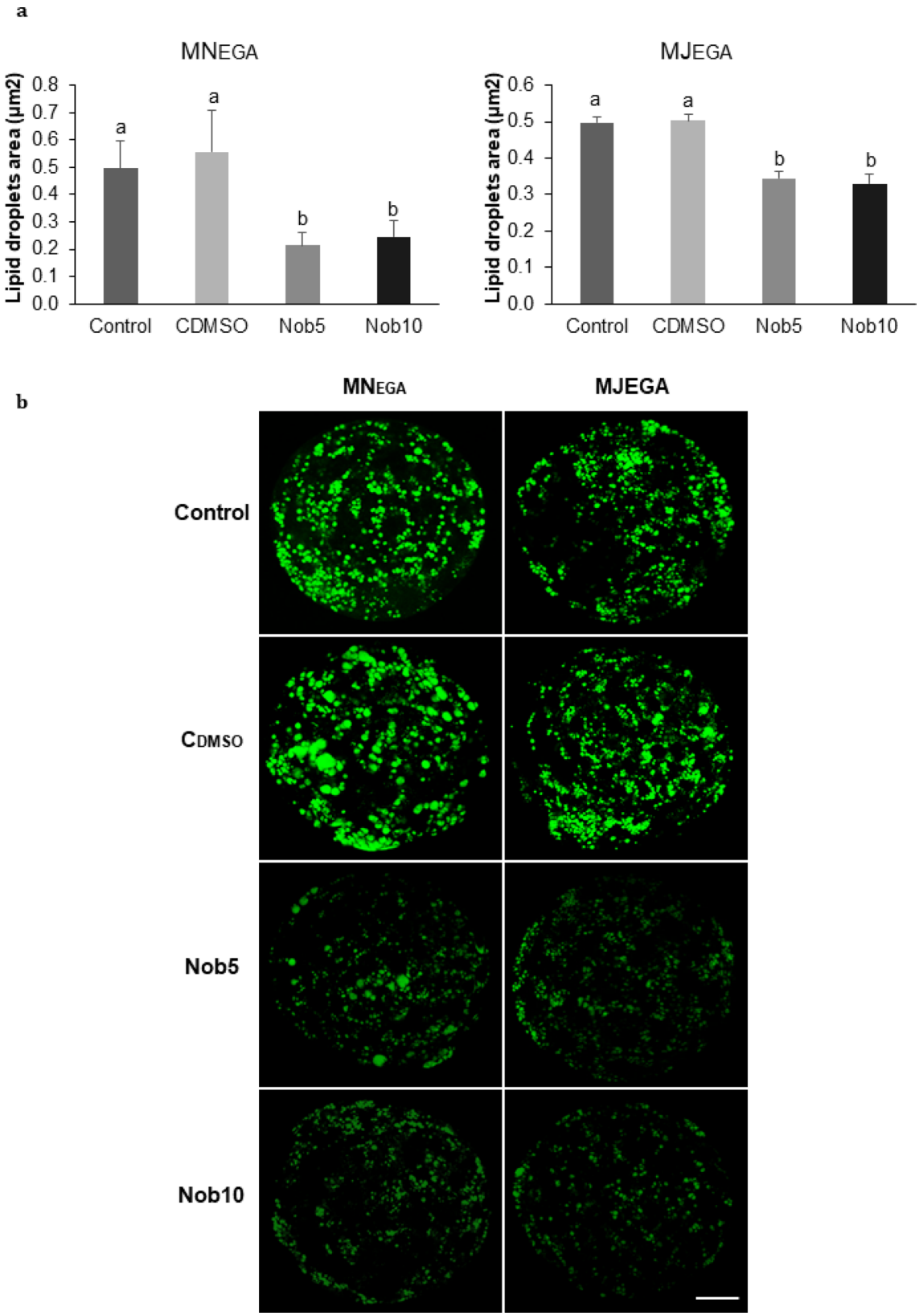

Figure 3. Nobiletin effect in blastocysts lipid content. (**a**) Quantification of the total area of lipid droplets (μm^2) in Day 7 blastocysts cultured in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}) during 21–54 hpi (MN_{EGA}: presumptive zygote to 8-cell stage) or during 54–96 hpi (MJ_{EGA}: 8- to 16-cell stage). Data are the mean ± s.e.m. Significant differences ($P < 0.001$) are indicated with different letters. (**b**) Representative fluorescence images of lipid droplets in Day 7 blastocysts from all experimental groups (Control, Nob5 Nob10, C_{DMSO}) in both phases (MN_{EGA} or MJ_{EGA}). Images were captured on 63× objective. Scale bar 50 µm.

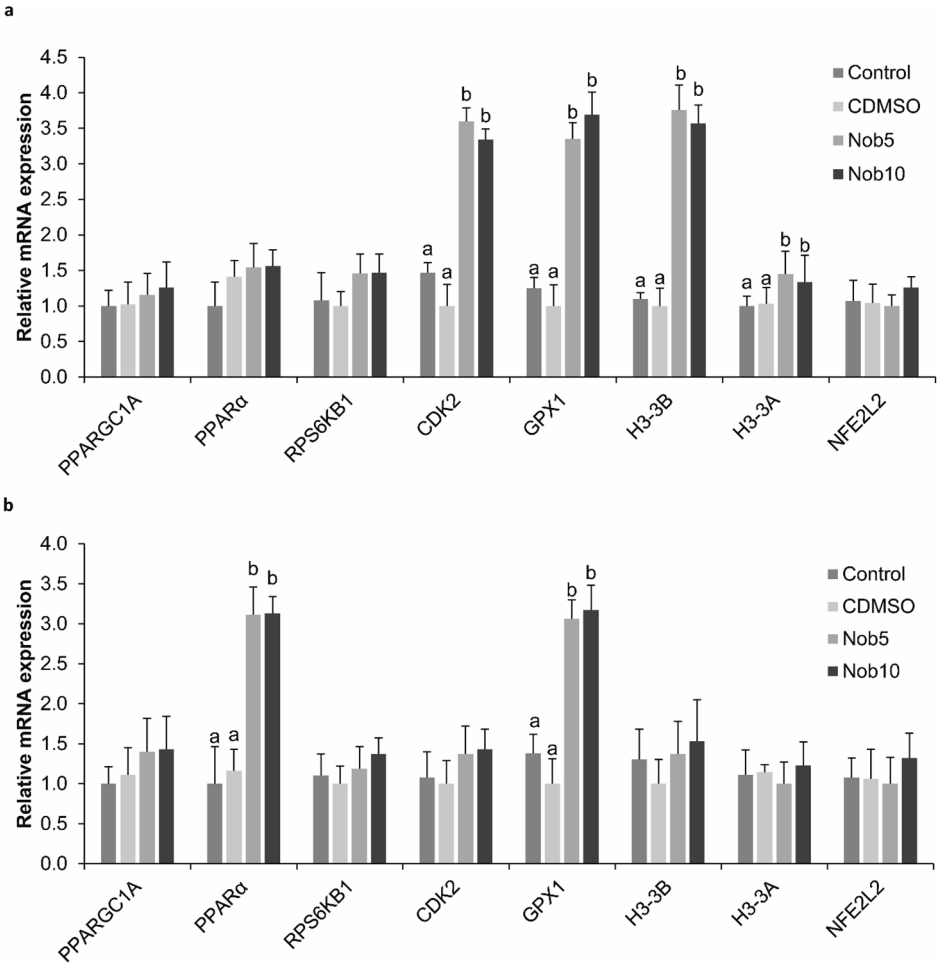

Figure 4. Relative mRNA transcript abundance of embryo development-related genes in in vitro produced embryos cultured during 21–54 h post insemination (MN_{EGA}: presumptive zygote to 8-cell stage) with or without nobiletin. (**a**) Relative abundance in 8-cell stage embryos cultured in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}) during MN_{EGA} phase. (**b**) Relative abundance in blastocysts from Control, Nob5, Nob10, and C_{DMSO} experimental groups from MN_{EGA} phase. The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as housekeeping genes. Data are the mean ± s.e.m. Different letters indicate significant difference ($P < 0.05$) between treatments.

and alter their efficiency to respond to oxidative phosphorylation[6]. Mitochondria also sense changes in redox potential and force embryos to adapt versus the decreased production of ATP by oxidative phosphorylation during the transition from morula to blastocyst[6,25]. Besides, some studies reported that changes in mitochondrial activity may affect the development of energetic metabolism in the embryo, in terms of availability of glucose, lipids, amino acids and DNA methylation[6,26]. Although the nobiletin action mechanism in mitochondria has not been fully elucidated, in a previous study we observed that increased oocyte mitochondrial activity was related to the cytoprotective effects of nobiletin and its intrinsic ROS-scavenging property[18]. Nevertheless, the effect in the blastocyst could be explained based on the fact that nobiletin is a hydrophobic compound, which easily penetrates through cell membranes directly affecting mitochondrial bioenergetics. Nobiletin can modify intramitochondrial proteins (e.g. acetylated proteins localized within the mitochondria in the brain of rats)[27] or alter the mitochondrial membrane potential by changing the activities of mitochondrial enzymes, like succinate dehydrogenase and cytochrome c oxidase as it has been demonstrated in human blood lymphocytes[28]. However, to verify if this mechanism occurs in bovine blastocysts, further investigation is necessary.

Lipid content is a crucial factor for early embryo development in vitro in bovine since energy metabolism is abnormal under such conditions, resulting in an excessive accumulation of lipids associated with reduced embryonic quality[29]. Lipids are stored in intracellular droplets and are metabolized via β-oxidation in the mitochondrial matrix. A large amount of lipid droplets increases the production of ATP necessary for the formation of blastocysts but this can affect its quality; thus, a lower number of lipid droplets in blastocysts is considered as a criterion of good quality embryos[16]. Our results showed for both EGA phases, nobiletin supplementation in culture medium reduced the amount of lipids in blastocysts. Furthermore, we analyze the expression of peroxisome proliferator-activated receptor alpha transcript (*PPARα),* belonging to one of the 3 key nuclear receptors

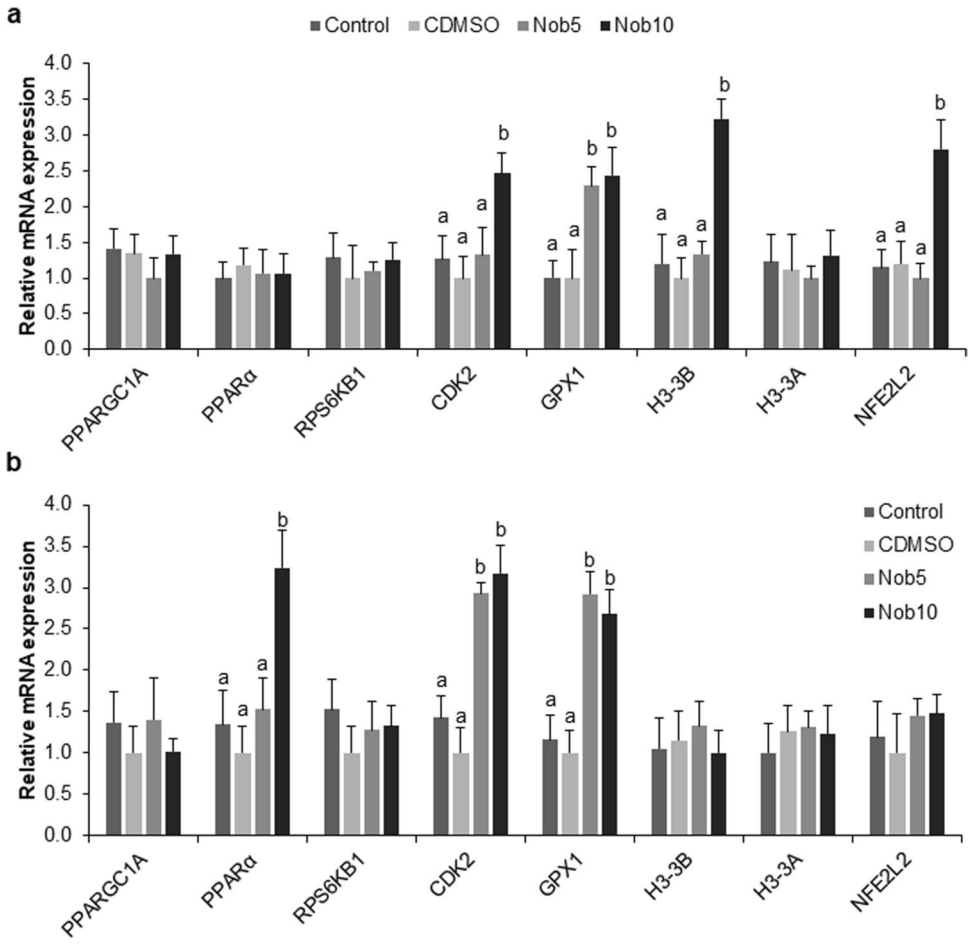

Figure 5. Relative mRNA transcript abundance of embryo development-related genes in in vitro produced embryos cultured during 54–96 hpi (MJ_{EGA}: 8-to 16-cell stage) with or without nobiletin. (**a**) Relative abundance in16-cell stage embryos cultured in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}) during MN_{EGA} phase. (**b**) Relative abundance in blastocysts from Control, Nob5, Nob10, and C_{DMSO} experimental groups from MJ_{EGA} phase. The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as housekeeping genes. Data are the mean ± s.e.m. Different letters indicate significant difference ($P < 0.05$) between treatments.

in the modulation of transcription for lipid metabolism-related genes[30]. *PPARα* was previously detected in cattle embryos and has been associated with embryo quality[31]. In our study, *PPARα* was significantly upregulated in blastocysts produced with both concentrations of nobiletin supplementation during MN_{EGA} phase or 10 µM of nobiletin supplementation during MJ_{EGA} compared to controls. These results together are in line with other studies which demonstrated that antioxidant supplementation in IVC medium, like crocetin[23] and L-carnitine[21], improved embryo quality by decreasing their lipid content. Regarding nobiletin, studies in mice showed its ability to reduce hepatic lipid accumulation, prevent lipoprotein overproduction and normalize insulin sensitivity when supplied in the diet[32]. Moreover, it has been demonstrated that nobiletin reduces lipid accumulation and regulates lipidic metabolism in hepatic cell lines[17,33]. There is evidence that nobiletin upregulates the expression of *PPARα* in white adipose tissue of mice[17]. An explanation for the reduction of lipids by nobiletin has been proposed indicating that full methoxylation of the A-ring of nobiletin seems to be the most optimal structure to express potent effects on modulating hepatic lipid metabolism via primarily suppressing lipoprotein secretion in HepG2 cells[33]. Therefore, it appears that the ability of nobiletin to reduce lipid content and improve mitochondrial activity in blastocysts may be related to the properties of its chemical structure that allows modulation of lipid metabolism and mitochondrial activity. Moreover, activation of *PPARα* by nobiletin could result in increased embryo lipid turnover through the β-oxidative pathway, preventing accumulation of lipoperoxides despite peroxisomal induction. Recent studies have shown that response of embryos to IVC involves a variety of metabolic factors that act as signals of extracellular and intracellular conditions to which the early embryos can adapt cell programming, signaling pathways, mitochondrial metabolism (mitochondrial production of Acetyl-Coenzyme A (Acetyl-Co A) and methyl groups, which are dependent on the availability of glucose, lipids and amino acids) or peroxisome proliferator-activated receptors (PPARs) in response to lipid content. These factors in the embryo are translated into effects on developmental speed or epigenetic modifications[6,34,35]. Consequently, these results

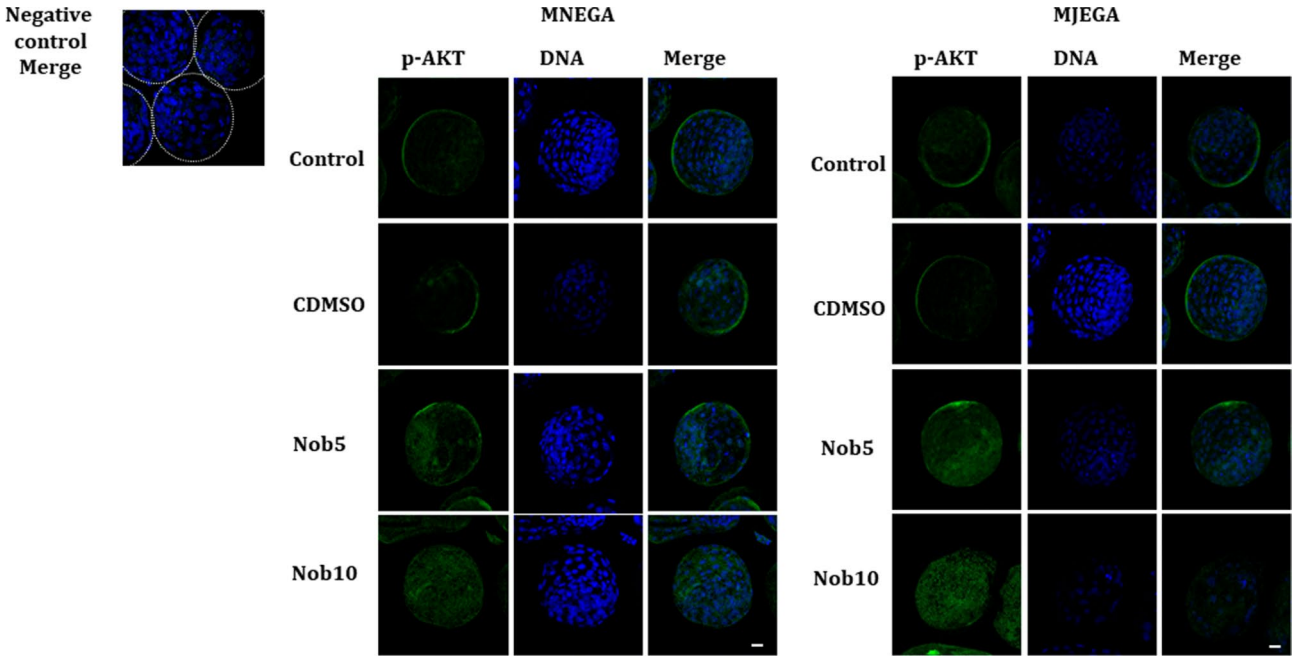

Figure 6. Nobiletin effect in the phosphorylated-AKT (p-AKT) in blastocysts. Representative images of immunofluorescence detection of p-AKT in in vitro produced bovine blastocysts cultured during 21–54 hpi (MN_{EGA}: presumptive zygote to 8-cell stage) or during 54–96 hpi (MJ_{EGA}: 8- to 16-cell stage) in SOF + 5% FCS (Control), supplemented or not with 5 (Nob5) or 10 µM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}). Positive staining for p-AKT proteins shown in green. Cell nuclei were counterstained with Hoechst stain (blue). Images were captured on 63× objective. Scale bar 20 µm.

can reinforce the antioxidant-defense role of nobiletin during early embryo development in vitro in bovine and could indicate an improvement of the quality of the produced blastocysts.

Embryo cell number is a parameter correlated with embryonic development and quality. Also, it has been reported for different cellular lines (MOLT-4, HUVEC, PC12D, K-N-SH cells) that nobiletin exert its activity by modulation of cell cycle progression[17]. We observed that regardless of EGA phase (MN_{EGA} and MJ_{EGA}), nobiletin supplementation in culture media increased the total cell number of produced blastocysts. This increase rate was similar to that observed with other antioxidants such as vitamin C[15] or crocetin[16,23], suggesting that nobiletin could directly stimulate the cell cycle during EGA and improve embryo quality.

To verify if the effects of nobiletin during MN_{EGA} or MJ_{EGA} were related to gene expression changes, we analyzed the expression of candidate genes for oxidative stress, embryo development and quality. Glutathione peroxidase (*GPX1*) and Nuclear Factor Erythroid 2-Like 2 (*NFE2L2*), are oxidative-stress-response-related genes. *GPX1*, considered the major antioxidant enzyme within the Glutathione peroxidase family, is ubiquitously expressed in the cytosol and also has been found in mitochondria[20]. Furthermore, *GPX1* acts as a scavenger of hydrophilic peroxide species, can be transformed into an enzymatically inactive cellular structural component, and protects cells against oxidative damage[20]. During in vitro production ROS generation increases and one of the defenses to counter excess ROS in the embryo is *GPX1*; therefore, *GPX1* overexpression has been positively linked with embryo quality[36,37]. In our study, gene expression analysis revealed the upregulation of *GPX1* in 8- and 16-cell embryos as well as in blastocysts produced with nobiletin supplementation during MN_{EGA} or MJ_{EGA} phases. A similar response has been reported in sheep and bovine embryos treated with other types of antioxidants like L-carnitine[21] or crocetin[16]. *NFE2L2* transcript (also known as *Nrf2*) is important for embryo tolerance to oxidative stress during EGA as well as for its competence for development[2]. PI3K/AKT pathway plays a role in regulating *NFE2L2* activation and is involved in the regulation of protein kinases, which may induce nuclear translocation[38]. Harris and Hansen[39], and Ghanem et al.[29] reported in mice that up-regulation of *NFE2L2* transcript may protect embryos from oxidative stress through preservation of intracellular redox states to ensure normal embryonic development. In the same line, our results showed the relative abundance of *NFE2L2* transcript increased in 16-cell stage embryos cultured with 10 µM nobiletin during MJ_{EGA}, while remained unaltered in 8-cell stage embryos as well as in blastocysts from both treatments. Moreover, data obtained in cancer cells of mice showed that *NFE2L2* mRNA levels were upregulated when nobiletin was supplemented in culture medium[40]. Taken together, these data suggest that nobiletin plays an antioxidant-defense role via distinct pathways during the different phases of early embryo development in vitro. However, it is necessary to confirm this antioxidant action by measuring ROS levels in the embryos.

Cyclin Dependent Kinase 2 (*CDK2*) is necessary for cell cycle progression, and is a major kinase that governs AKT phosphorylation, while it also participates on EGA[41]. In our study, *CDK2* mRNA expression was upregulated in 8-cell (MN_{EGA}), and blastocysts (MJ_{EGA}) cultured with 5 µM or 10 µM of nobiletin as well as in 16-cell (MJ_{EGA}) cultured with 10 µM of nobiletin. This is in line with previous data obtained in bovine embryos

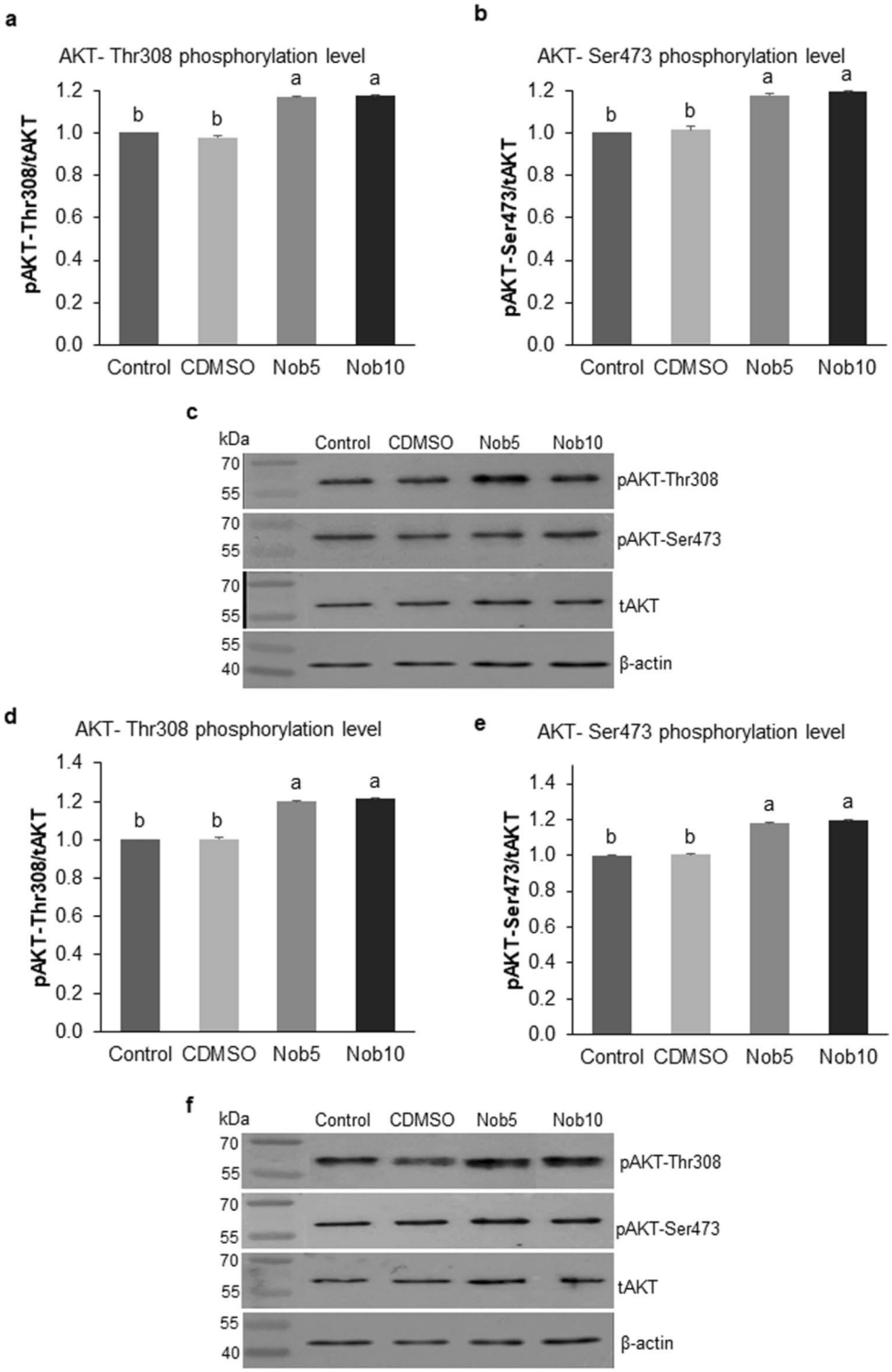

Figure 7. Nobiletin during MN_{EGA} or MJ_{EGA} increases AKT phosphorylation in blastocysts. (**a**,**b**) Quantification of phosphorylation levels of pAKT-Thr308/tAKT and pAKT-Ser473/tAKT in in vitro produced bovine Day 7 blastocysts cultured during 21–54 hpi (MN_{EGA}: presumptive zygote to 8-cell stage) in SOF + 5% FCS (Control), supplemented or not with 5 μM (Nob5) or 10 μM (Nob10) nobiletin or with 0.03% dimethyl sulfoxide (C_{DMSO}). (**c**) Western blot images showing the expression of pAKT-Thr308, pAKT-Ser473, (t)AKT and β-actin in Day 7 blastocysts from MN_{EGA} phase. (**d**,**e**) Quantification of phosphorylation levels of pAKT-Thr308/tAKT and pAKT-Ser473/tAKT in in vitro produced bovine Day 7 blastocysts cultured during 54–96 hpi (MJ_{EGA}: 8- to 16-cell stage) in Control, Nob5, Nob10, and C_{DMSO}. (**f**) Western blot images showing the expression of pAKT-Thr308, pAKT-Ser473, (t)AKT and β-actin in Day 7 blastocysts from MJ_{EGA} phase. Data were normalized relative to the abundance of β-actin and p-AKT phosphorylation levels. Samples derive from the same experiment and gels were processed in parallel. Cropped western blot membrane images are shown here, while full-length blots are presented in Supplementary Figure S1. Data are the mean ± s.e.m. Different letters indicate significant difference ($P < 0.05$) between treatments.

that showed changes in the levels of transcription in genes associated with cell cycle and observed an increase in *CDK2* expression during early embryo development (8 and 16-cell embryos, and blastocysts)[42]. Conversely, studies using nobiletin in cancer cells (U87, Hs683) showed a decrease in *CDK2* expression[17,43]. Hence, nobiletin seems to respond differently depending on the cell type.

Histone H3.3 is encoded by H3.3 histone A (*H3-3A*) and H3.3 histone B (*H3-3B*) genes and is related to DNA synthesis and integrated into embryonic nucleosomes to mark genes for subsequent expression in development[44]. We observed that supplementation of 5 or 10 µM nobiletin during MN_{EGA}, increased the expression of *H3-3B* and *H3-3A* genes in 8- cell embryos while supplementation of 10 µM nobiletin during MJ_{EGA} increased the expression of *H3-3B* gene in 16-cell embryos. This is in corroboration with results from a recent study were characterization of the expression of both genes that encode *H3.3* (*H3-3A and H3-3B*) was performed in early bovine embryos, demonstrating that *H3-3B* mRNA is very abundant throughout early embryogenesis, being two to three times higher than *H3-3A* mRNA during the major wave of EGA[45]. Additionally, a higher abundance of *H3-3B* compared to *H3-3A* was found in mouse embryos[46], suggesting that the protein encoded by *H3-3B* gene may be critical for initiating the transcription of embryonic genes during EGA. As mentioned above, EGA is crucial for further embryo development and regulated by several important factors[47]. One crucial factor is histone modification, including methylation and acetylation[48].Likewise, Acetyl-Co A is a central metabolite linking glucose oxidation and long-chain fatty acid or cholesterol synthesis, providing energy and materials for cell growth and proliferation. Furthermore, Acetyl-Co A, as a donor of an acetyl group, can be utilized by histone acetyltransferases for histone acetylation[49]. A recent study showed that Acetyl-CoA synthases are essential for maintaining histone acetylation under metabolic stress during EGA in pigs and they corroborated that β-oxidation is crucial for porcine embryo development by contributing to energy metabolism and histone acetylation[50]. This suggests one more time that nobiletin could prefer the β-oxidation pathway as an energy production mechanism.

During in vitro development, embryos have a series of metabolic factors that are required in proliferation, differentiation, and survival of cells[13,51]. In this context, the quality of the embryos produced in vitro depends on many factors, among them the expression of different genes, as we have shown in this study. Gene expression depends of different signaling pathways that play important roles in the formation of the blastocyst, for example, PI3K/AKT[12,13,51]. AKT regulates cellular processes such as glucose metabolism, transcription, cellular growth and proliferation[51]. In blastocysts, AKT inhibition alters their development and AKT activation triggers the differentiation and migration of trophoblast cells[14,52]. Other studies showed that the AKT appear to have an important role in early embryonic development, in double-knockout mice deletion of any of the AKT isoforms leads to embryonic death or exhibiting more severe phenotype and earlier lethality[53]. In addition, PI3K/AKT regulates the development of preimplantation embryo by mediating the effects of autocrine factors[54]. Previous studies in cell lines have shown that nobiletin can act through various signaling pathways, including AKT[17]. However, as far as we know, nobiletin action on AKT pathway in bovine blastocysts produced in vitro is unknown. In this study, we established the presence of the AKT pathway in bovine blastocysts. Based on immunofluorescence images, p-AKT protein appears to be predominantly localized in the cytoplasm of embryos cultured with 5 and 10 µM nobiletin during MN_{EGA} and MJ_{EGA}, suggesting constant stimulation of this pathway during the preimplantation period. Expression of the AKT protein and its phosphorylation status were confirmed by western blot analysis of bovine blastocysts produced with or without nobiletin supplementation during MN_{EGA} or MJ_{EGA} phases. Similar results were found by Ashry et al.[14], who investigated the relationship between AKT signaling and the embryotrophic actions of follistatin, and indicated that it plays an important role in the regulation of AKT signaling in early bovine embryos. Together, these results suggest that nobiletin is associated with increased AKT phosphorylation and, as it has been shown in cell lines studies, nobiletin has the ability to interact with this pathway, and regulate specific genes. In our study increased AKT phosphorylation might be related to the increase in the production and the expression of genes that favor the progression of the cell cycle (*CDK2*) and to the improvement in embryo quality by increasing mitochondrial activity and genes related to oxidative stress (*NFE2L2*), and reducing lipid content (*PPARα*). However, further studies are needed to fully elucidate its mechanism of action in early embryos.

In conclusion, nobiletin supplementation during MN_{EGA} or MJ_{EGA} has a positive effect on preimplantation bovine embryonic development in vitro by increasing blastocyst production and also corroborates on the increase in transcription level of genes related to cell division. Besides, this effect is reflected on the blastocysts quality improvement by (i) stimulating mitochondrial activity and expression of genes related to the protection of oxidative stress, and (ii) reducing the cytoplasmatic accumulation of lipids and promoting the expression of genes that regulate lipid metabolism. In addition, these positive responses of nobiletin on embryonic development and quality of the produced blastocysts in vitro could be modulated by the activation of AKT signaling pathway (Fig. 8). Therefore, nobiletin could constitute a suitable supplement to overcome oxidative stress in bovine IVP and improve ARTs in mammals.

Methods

Unless stated otherwise, all reagents were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA).

Oocyte collection and maturation. Immature cumulus-oocyte complexes (COCs) were obtained by aspirating follicles (2–8 mm) from the ovaries of mature heifers and cows collected from a local abattoir. COCs (homogeneous cytoplasm and intact CCs) were selected and matured in four-well dishes (Nunc, Roskilde, Denmark) in 500 µL maturation medium (TCM-199), supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/mL epidermal growth factor (EGF), in groups of 50 COCs per well for 24 h at 38.5 °C and an atmosphere of 5% CO_2 in the air with maximum humidity[55].

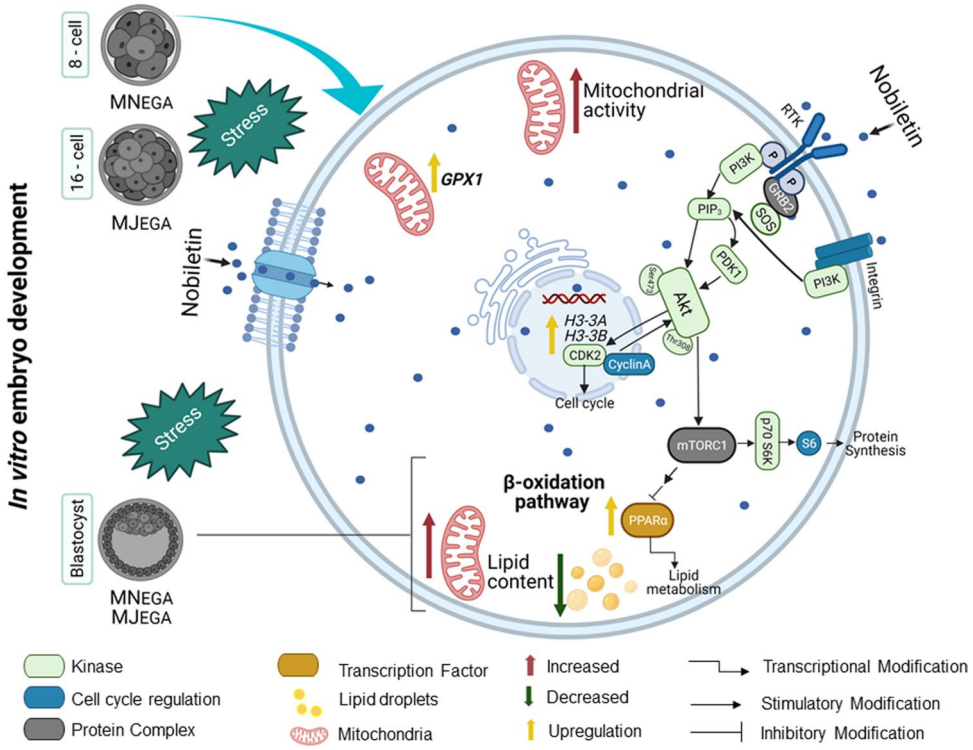

Figure 8. Schematic diagram illustrating the effect of nobiletin on bovine embryo development during the two EGA phases (MN_{EGA} and MJ_{EGA}). Nobiletin a class of polymethoxylated flavone, is easily absorbed across the cytoplasmic membranes due to its structure and lipophilic nature[17], but the specific mechanism of action is not clear yet. Nobiletin supplementation during EGA has a positive effect on preimplantation bovine embryonic development, also increase the abundance of *CDK2* (cell cycle progression), *H3-3A*,*H3-3B* (development) and *GPX1* (oxidative stress) transcripts in 8-cells and 16-cells embryos. In addition, the nobiletin effect in the produced blastocysts was reflected by stimulating mitochondrial activity, decreasing the cytoplasmatic accumulation of lipids and promoting the expression of genes that regulate lipid metabolism and protect against oxidative stress. Besides, these positive responses of nobiletin on embryonic development and quality of the produced blastocysts in vitro could be modulated by activation of the AKT signaling pathway. In our study increased AKT phosphorylation might be related to the increase in the production and the expression of genes that favor the progression of the cell cycle (*CDK2*) and reducing lipid content (*PPARα*). However, further studies are needed to fully elucidate its mechanism of action in early embryos. Figure created with BioRender.com.

Sperm preparation and in vitro fertilization (IVF). IVF was performed as described previously[56]. Briefly, frozen semen straws (0.25 mL) from an Asturian Valley bull previously tested for IVF were thawed at 37 °C in a water bath for 1 min and centrifuged for 10 min at 280×g through a gradient of 1 mL of 40% and 1 mL of 80% Bovipure (Nidacon Laboratories AB, Göthenborg, Sweden) according to the manufacturer´s instructions. The sperm pellet was isolated and washed in 3 mL of Boviwash (Nidacon Laboratories AB) by centrifugation at 280×g for 5 min. The pellet was re-suspended in the remaining 300 µL of Boviwash. The final concentration of spermatozoa was adjusted to 1×10^6 spermatozoa/mL. Gametes were co-incubated for 18–22 h in 500 µL fertilization medium (Tyrode's medium) with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate and 6 mg/mL fatty acid-free bovine serum albumin (BSA) supplemented with 10 mg/mL heparin sodium salt (Calbiochem) in four-well dishes in groups of 50 COCs per well in an atmosphere of 5% CO_2 in the air with maximum humidity at 38.5 °C.

In vitro culture of presumptive zygotes. At approximately 21 hpi, a total of 7237 (3398 for MN_{EGA} phase and 3839 for MJ_{EGA} phase) presumptive zygotes were denuded of cumulus cells (CCs) by vortexing for 3 min and then cultured in groups of 50 in a four-well dish containing 500 µL per well of culture medium (synthetic oviductal fluid (SOF);[57] supplemented with 5% (v/v) FCS, 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 µL/mL basal medium eagles (BME) amino acids, 10 µL/mL minimum essential medium (MEM) amino acids and 1 µg/mL phenol red, in the presence (MN_{EGA}) or absence (MJ_{EGA}) of nobiletin (MedChemExpress, MCE, Sweden) or with 0.03% dimethyl sulfoxide (DMSO vehicle for nobiletin dilution). At 54 hpi those embryos that reached the 8-cell stage were selected and randomly cultured in groups of 50 in SOF + 5% FCS until Day 8 (MN_{EGA}) or in presence of nobiletin or DMSO until 96 hpi (MJ_{EGA}). At 96 hpi those embryos that reached the 16-cell stage were selected and randomly cultured in groups of 50 in SOF + 5% FCS until Day 8 (MJ_{EGA}) (see

'Experimental design' described below for more details). Culture took place at 38.5 °C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

Assessment of embryo development and quality

Embryo development. Developmental rate was recorded at 54 hpi from MN_{EGA} and MJ_{EGA} (≥ 8-cell) and 96 hpi from the MJ_{EGA} phase (≥ 16-cell). For both phases, cumulative blastocyst yields were recorded at Day 7, and 8 pi under a stereomicroscope.

Embryo quality: mitochondrial activity measurement, lipid content quantification and total cell number of blastocysts. Day 7 blastocysts (~ 30 per group) were simultaneously evaluated regarding mitochondrial activity, the number of lipid droplets, and total cell number. Blastocysts from each treatment were first suspended in 100 µL phosphate-buffered saline (PBS) without calcium or magnesium supplemented with 0.1% polyvinylpyrrolidone (PVP). Next, blastocysts were equilibrated for 15 minutes in culture media supplemented with 5% FCS and then incubated for 30 min at 38.5 °C in 400 nM MitoTracker DeepRed (Molecular Probes, Eugene, USA) for mitochondrial activity; blastocysts were then fixed in 4% paraformaldehyde (PF) for 30 min at room temperature. For lipid content analysis, fixed blastocysts were permeabilized with 0.1% saponin for 30 min and stained for 1 h with 20 µg/mL Bodipy 493/503. For analysis of total cell number, blastocysts were stained with Hoechst 33342 (10 µg/mL) for 30 min, washed in PBS + 0.1% PVP three times for 5 minutes each, and then mounted in 3.8 µL mounting medium between a coverslip and a glass slide which was sealed with nail polish. Slides were examined using a laser-scanning confocal microscope (Leica TCS SP2) equipped with an argon laser excited at 488 nm and with an emission spectrum of 500–537 nm for visualization of lipid droplets. For mitochondria, we used excitation and emission set at 644 nm and 625–665 nm, respectively. All images were captured using the same parameters, performing sequential acquisition.

For the assessment of mitochondrial activity, the fluorescence signal intensity (pixels) was quantified. Serial sections of 5 µm were made for each blastocyst and a maximum projection was accomplished for each one. Images obtained were evaluated using the ImageJ program (NIH; ImageJ version 1.52k software (http://rsbweb.nih.gov/ij/)). After selection using the freehand selection tool, each blastocyst was measured to determine its area and its integrated density (IntDen), which corresponds to pixel intensity. Also, the background fluorescence of an area outside the blastocyst was measured. Fluorescence intensity in each blastocyst was determined using the following formula: Relative fluorescence = IntDen − (area of selected blastocyst x mean fluorescence of background readings). Fluorescence intensities are expressed in arbitrary units (a.u.).

The lipid quantification in blastocysts was obtained by analysis of the total area of lipids in each blastocyst. We captured three images of each blastocyst: one in the middle of the blastocyst (the image with the largest diameter) and the other two in the middle of the resulting halves. We used a 63× objective at a resolution of 1024 × 1024 and images were analyzed using the 'nucleus counter' tool, set to detect, distinguish, and quantify droplet areas with the ImageJ program. For blastocysts, lipid quantity was corrected by total embryo area, to account for varying blastocyst sizes. After verification of a significant correlation ($r^2 = 0.84$ and $P < 0.0001$ by Pearson's correlation test) between lipid quantity of three sections in 30 blastocysts (10 per group) we chose the section with the largest area per blastocyst to be analyzed[58]. Simultaneously, the total number of cells per blastocyst was determined by counting the Hoechst stained cells under an epifluorescence microscope (Nikon 141731) equipped with a fluorescent lamp (Nikon HB-10104AF) and UV-1 filter.

Embryos at 8- and 16-cell stage and blastocysts for gene expression analysis. Gene expression analysis was performed using 3 pools of 10 embryos at 8-cell (MN_{EGA}); 16-cell (MJ_{EGA}); and Day 7 blastocysts of both phases (MN_{EGA} and MJ_{EGA}) per treatment group: Control, C_{DMSO}, Nob5, and Nob10. Poly(A) RNA was extracted using the Dynabeads mRNA Direct Extraction Kit (Ambion; Thermo Fisher Scientific) with minor modifications[59]. Immediately after poly(A) RNA extraction, reverse transcription (RT) was performed using a Moloney murine leukemia virus (MMLV) Reverse Transcriptase 1st-Strand cDNA Synthesis Kit according to the manufacturer's instructions (Epicentre Technologies Corp, Madison, WI, USA). Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure, allowing Poly(T) random primers and Oligo dT annealing, and the RT mix was then completed by adding 0.375 mM dNTPs (Biotools, Madrid, Spain), 6.25 U RNAsin RNAse inhibitor (Promega, Madison, WI, USA), MMLV HP RT 10x reaction buffer, 5 mM DTT and 5 U MMLV high-performance reverse transcriptase (Epicentre Technologies Corp, Madison, WI, USA). Samples were incubated at 25 °C for 10 min, and then at 37 °C for 60 min, to allow the RT of RNA, and finally at 85 °C for 5 min to denature the enzyme. All mRNA transcripts were quantified in duplicate using a Rotorgene 6000 Real-Time Cycler (Corbett Research, Sydney, Australia). RT–quantitative polymerase chain reaction (qPCR) was performed by adding a 2 µL aliquot of each cDNA sample (~ 60 ng μL^{-1}) to the PCR mix (GoTaq qPCR Master Mix, Promega) containing specific primers to amplify the genes of interest. Primer sequences are provided in Supplementary Table S4. The selection of genes to be evaluated was carried out considering the expression of key genes in preimplantation embryonic development. All primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries when possible. For quantification, RT-qPCR was performed as described previously[60]. The PCR conditions were tested to achieve efficiencies close to 1. Relative expression levels were quantified by the comparative cycle threshold (CT) method[61]. Values were normalized using two housekeeping genes (*H2AFZ* and *ACTB*) selected according to previous studies[56,62], while their stabilities were evaluated using the geNorm software for microsoft[63,64], ranking the genes based on the internal control gene stability parameter M. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a dou-

bling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the mean CT value of the two housekeeping genes from the CT value of the gene of interest in the same sample. The calculation of ΔΔCT involved using the highest treatment ΔCT value (i.e. the treatment with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold-changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT.}$

Immunofluorescence of phospho-AKT (p-AKT) in blastocysts. Immunolocalization of p-AKT was performed according to López-Cardona et al.[65] with minor modifications. Day 7 blastocysts (n = 10 per group) were washed twice with PBS + 0.1% PVP and fixed in 4% PF for 10 min at room temperature. Next, they were permeabilized by incubation in PBS with 10% FCS and 1% Triton X-100 for 45 min at room temperature. After permeabilization, blastocysts were incubated overnight at 4 °C in PBS + 0.1% PVP and 5% FCS and 1:100 rabbit polyclonal antibody against p-AKT (Thr308/Ser473) (D9E) XP® Rabbit mAb (Cell Signaling Technology, #4060). Following incubation, blastocysts were washed twice in PBS + 0.1% PVP and incubated in PBS supplemented with 5% FCS and 1:250 goat anti-rabbit polyclonal antibody Alexa Fluor 488-conjugate (Molecular Probes, Eugene, OR, USA), for 2 h at room temperature followed by washing again three times in PBS + 0.1% PVP. In all cases, nuclei were stained with Hoechst 33342 (10 µg/mL). Finally, blastocysts were mounted in microdrops with Fluoromount G (EMS, Hatfield, UK) and examined by confocal microscopy (Leica TCS-SPE). Negative control was prepared to omit the primary antibody before adding the secondary antibody.

Western blot of AKT in blastocysts. The western blot analysis was performed as described previously by Ashry et al.[66] with minor modifications. Day 7- 8 blastocysts (n = 20 blastocysts/group, n = 3 replicates/EGA phase) were lysed in 1 × RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris [pH 7.6]), supplemented with 1 × protease, phosphatase Inhibitor Cocktail (Roche, Basel, Switzerland), for 1 h at 4 °C. The samples were mixed with 1 × sample buffer and then denatured at 95 °C for 10 min. Proteins were resolved by SDS-PAGE (12% acrylamide gel loading 45 µL of total protein per well) and transferred onto a nitrocellulose membrane. After the transfer, membranes were blocked for 30 min in 3% BSA in PBS + 0.1% Tween-20 (PBS-T) at room temperature, and was incubated overnight at 4 °C with a total (t)AKT rabbit polyclonal antibody [1:1000 (Vol:Vol), Cell Signaling Technology, #9272S]; or a p-AKT (Thr308) polyclonal antibody [1:1000 (Vol:Vol), Cell Signaling Technology, #9275S]; or a p-AKT (Ser473) polyclonal antibody [1:1000 (Vol:Vol), Cell Signaling Technology, #9271S]. Then, incubation with the secondary antibody goat anti-rabbit IgG-HRP [1:2500 (Vol:Vol), Cell Signaling Technology, #7074S] was conducted for 2 h at room temperature revealed by Enhanced Chemiluminescence kit (RPN2109, ECL™, Amersham GE Healthcare) and detected by an ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare Life Sciences, USA, 29005063). The monoclonal anti-β-actin–peroxidase antibody produced in mouse was used as the loading control.

Membranes were probed sequentially with primary p-AKT (Thr308), p-AKT (Ser473) and (t)AKT antibodies. For this purpose, after detection of an antibody membranes were stripped by washing extensively in TBS-T, three times for 10 minutes each, and repeating the blocking step, and then the membranes are re-probed with the next antibody. After detection of (t)AKT, membranes were stripped and re-probed with anti-β-actin–peroxidase antibody. In all cases, intensities of protein bands (optical density (OD)) were quantified by ImageJ software and the relative abundance of each protein was normalized to the total–actin expression in the corresponding lane and phosphorylation level was expressed as phosphorylated (p) AKT/(t) AKT. The ratio of the OD of the protein concerned (AKT/p-AKT) in relation to actin is presented in the form of bar charts.

Experimental design. *Experiment 1: effect of nobiletin on early embryo development in vitro.* In this experiment the effect of nobiletin supplementation on embryo development during two developmental periods: (a) MN_{EGA}: from 2-cell to 8-cell stage, minor EGA phase; and (b) MJ_{EGA}: from 8-cell to 16-cell stage, major EGA phase was determined by evaluating the cleavage rate at 54 hpi and blastocysts yield at Days 7 and 8 (Fig. 9).

For this purpose, presumptive zygotes/embryos from 2- to 8- cell stage (MN_{EGA}: 21-54 hpi) or embryos from 8- to 16-cell stage (MJ_{EGA}: 54–96 hpi) were cultured in SOF + 5% FCS alone (Control: n = 730 and 621 for MN_{EGA} and MJ_{EGA} respectively) or supplemented with 5, 10 or 25 µM nobiletin (Nob5: n = 757 and 518; Nob10: n = 695 and 553; and Nob25: n = 521 and 424 for MN_{EGA} and MJ_{EGA} respectively), or 0.03% DMSO (C_{DMSO}: n = 695 and 622 for MN_{EGA} and MJ_{EGA} respectively). For MJ_{EGA} phase groups, embryo culture until 8-cell stage (21-54 hpi) was performed in SOF + 5% FCS. At 54 hpi (MN_{EGA} - Control: n = 388; Nob5: n = 386; Nob10: n = 352; Nob25: n = 254; C_{DMSO}: n = 368) or 96 hpi (MJ_{EGA}- Control: n = 331; Nob5: n = 315; Nob10: n = 347; Nob25: n = 210; C_{DMSO}: n = 331), embryos that reached the 8- or 16- cell stage, respectively, were transferred to SOF + 5% FCS and cultured until Day 8, maintaining the different experimental groups separately (Fig. 9). Embryos were cultured in groups of 50 under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C.

Considering that during the experiment it was necessary to preselect the embryos at different stages of development (≥ 8 cells and ≥ 16 cells), the developmental parameters were calculated as follows: (I) developmental rate at 54 hpi: percentage of presumptive zygotes that developed to the 8-cell stage; (II) developmental rate at 96 hpi: percentage of selected 8-cell embryos at 54 hpi that developed to the 16-cell stage; and (III) blastocyst yield: percentage of selected 8-cell embryos (54 hpi) or 16-cell embryos (96 hpi) that continued in culture and developed to the blastocyst stage at Day 7 and 8. A representative number of 8-cell (MN_{EGA}), 16-cell (MJ_{EGA}), and Day 7 blastocysts from both phases for each experimental group were frozen in liquid nitrogen (LN_2) in three groups of 10 and stored at − 80 °C for gene expression (See Experiment 2). Additionally, Day 7 blastocysts from both phases were selected to (i) evaluate quality (See Experiment 2); (ii) immunolocalization of p-AKT or (iii) for western blot analysis (See Experiment 3).

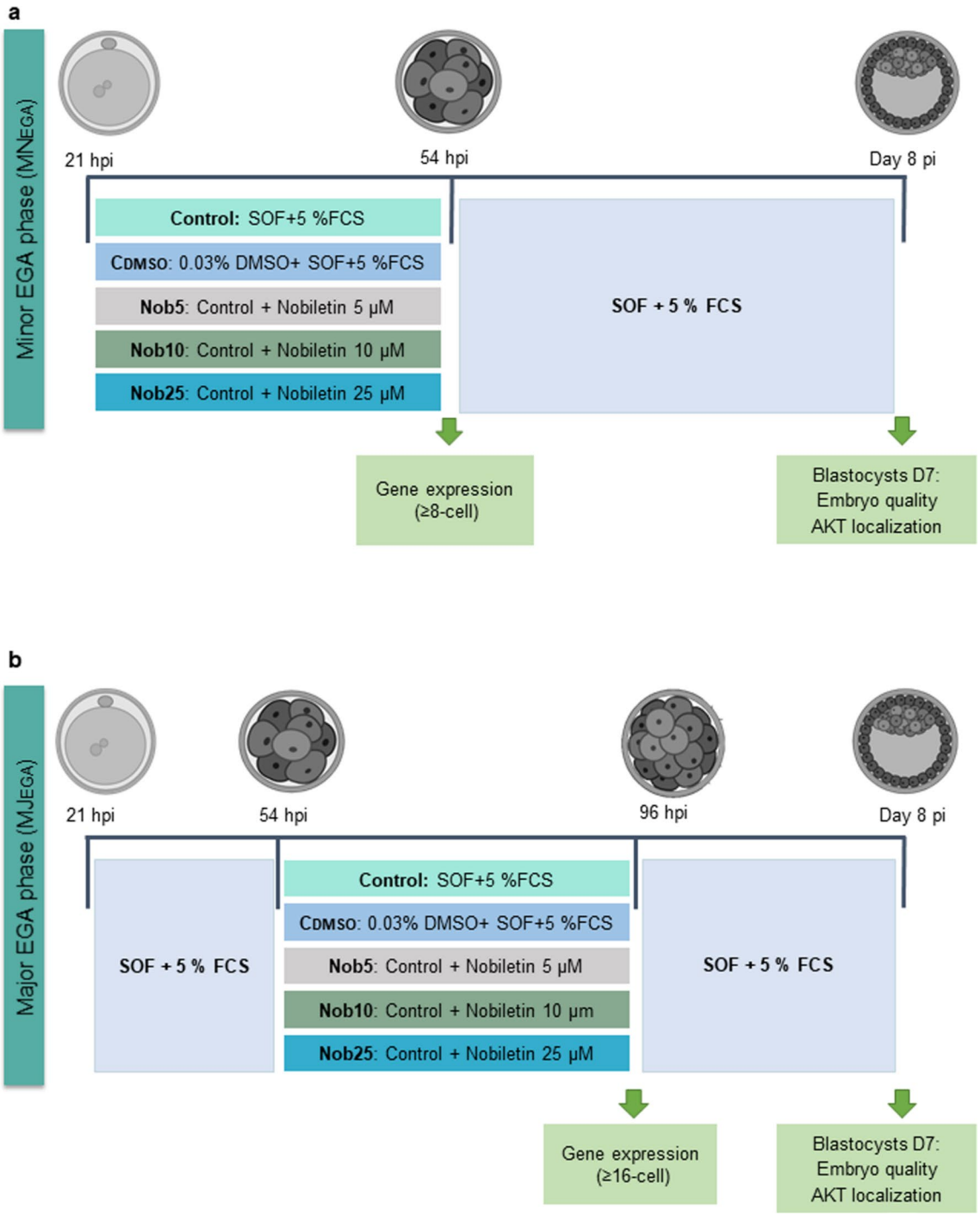

Figure 9. Experimental design. (**a**) MN_{EGA}: Presumptive zygotes were cultured during the minor EGA phase (2- to 8-cell stage: 21–54 hpi) in synthetic oviductal fluid (SOF) with 5% fetal calf serum (FCS) (Control); supplemented or not with 5, 10 and 25 µM nobiletin (Nob5, Nob10 and Nob25, respectively), or with 0.03% dimethyl sulfoxide [control for DMSO vehicle for nobiletin dilution (C_{DMSO})]. At 54 hpi, embryos that reached the 8-cell stage were transferred to SOF + 5% FCS and cultured until Day 8 maintaining each experimental group separately. (**b**) MJ_{EGA}: Presumptive zygotes were cultured in SOF + 5% FCS (Control) until 54 hpi. At 54 hpi, embryos that reached the 8-cell stage were cultured during the major EGA phase (8-cell to 16-cell stage: 54–96 hpi) in SOF + 5% FCS supplemented or not with Nob5, Nob10 and Nob25, or with 0.03% dimethyl sulfoxide (C_{DMSO}). At 96 hpi, embryos that reached the 16-cell stage were transferred to SOF + 5% FCS and cultured until Day 8 maintaining each experimental group separately.

Twelve and ten replicates for MN_{EGA} and MJ_{EGA} phases, respectively, were performed under the same assay conditions.

Only the experimental groups that showed higher blastocyst yield in this experiment (Nob5 and Nob10) in comparison with both control groups (Control and C_{DMSO}) were used for experiments 2 and 3.

Experiment 2: effect of nobiletin on the quality of in vitro produced blastocysts. To evaluate blastocyst quality of embryos produced in vitro with or without nobiletin supplementation during MN_{EGA} or MJ_{EGA}, a representative number of Day 7 blastocysts (n ≈ 30 per group/Experiment 1) were stained with MitoTracker DeepRed, Bodipy and Hoescht to evaluate mitochondrial activity (intensity recorded in arbitrary units (a.u)), lipid content (lipid droplet area in μm^2) and total cell numbers, respectively. Blastocysts were examined using a laser-scanning confocal microscope or an epifluorescence microscope and images obtained were evaluated using the ImageJ program.

To evaluate if nobiletin induces changes in the expression levels of genes related to embryo development and quality, three independent pools of 10 embryos per stage (8-cell, 16-cell, and blastocyst) obtained from each experimental group cultured with or without nobiletin during MN_{EGA} or MJ_{EGA} (Experiment 1), were used for gene expression analysis by qRT-PCR according to the procedures described above.

The selected genes have been linked to embryonic development and are essential in cell proliferation, differentiation, and embryo quality, such as *PPARG* coactivator 1 alpha (*PPARGC1A*); Peroxisome Proliferator-Activated Receptor Alpha (*PPARα*); Ribosomal Protein S6 Kinase Beta-1 (*RPS6KB1*); Cyclin Dependent Kinase 2 (*CDK2*); H3 Histone Family Member 3B (*H3-3B*) and H3 Histone Family Member 3A (*H3-3A*), including Nuclear Factor Erythroid 2-Like 2 (*NFE2L2*) and Glutathione Peroxidase 1 (*GPX1)* related with oxidative stress.

Experiment 3: nobiletin effect on the AKT pathway in blastocysts produced in vitro. To assess if nobiletin can interact with AKT pathway during in vitro embryo development, Day 7 blastocysts (n = 10 - Experiment 1) from each group were stained with p-AKT (Thr308/Ser473) for immunolocalization. To evaluate the phosphorylation level of AKT (Thr308 and Ser473), Day 7 blastocyst (n = 60 - Experiment 1) from each group were frozen in LN_2 for western blot analysis.

Statistical analysis. All statistical tests were performed using the software package SigmaStat (Systat Software Inc., San Jose, CA, USA). Cleavage rate, blastocyst yield, mitochondrial activity, lipid content, number of cells per blastocyst, relative mRNA abundance levels, and AKT phosphorylation level, were normally distributed with homogeneous variance, so one-way analysis of variance (ANOVA) with arcsine data transformation, followed by Tukey´s test, was performed to evaluate the significance of differences between groups. The correlation analysis for lipid quantification in blastocysts was determined by Pearson's correlation coefficient test. Values were considered significantly different at $P < 0.05$. Unless otherwise indicated, data are presented as the mean ± s.e.m.

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References

1. Rizos, D., Fair, T., Papadopoulos, S., Boland, M. P. & Lonergan, P. Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. *Mol. Reprod. Dev.* **62**, 320–327 (2002).
2. Gad, A., Schellander, K., Hoelker, M. & Tesfaye, D. Transcriptome profile of early mammalian embryos in response to culture environment. *Anim. Reprod. Sci.* **134**, 76–83 (2012).
3. Van Eetvelde, M., Heras, S., Leroy, J. L. M. R., Van Soom, A. & Opsomer, G. The Importance of the Periconception Period: Immediate Effects in Cattle Breeding and in Assisted Reproduction Such as Artificial Insemination and Embryo Transfer. in *Periconception in Physiology and Medicine* (eds. Fazeli, A. & Holt, W. V.) 41–68 (Springer International Publishing, 2017). https://doi.org/10.1007/978-3-319-62414-3_3.
4. Rizos, D., Maillo, V., Sánchez-Calabuig, M.-J. & Lonergan, P. The Consequences of Maternal-Embryonic Cross Talk During the Periconception Period on Subsequent Embryonic Development. in *Periconception in Physiology and Medicine* (eds. Fazeli, A. & Holt, W. V.) 69–86 (Springer International Publishing, 2017). https://doi.org/10.1007/978-3-319-62414-3_4.
5. Rizos, D. *et al.* Consequences of in vitro culture conditions on embryo development and quality. *Reprod. Domest. Anim.* **43**, 44–50 (2008).
6. Cagnone, G. & Sirard, M.-A. The embryonic stress response to in vitro culture: insight from genomic analysis. *Reproduction* **152**, R247–R261 (2016).
7. Gutierrez-Adán, A. *et al.* Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Mol. Reprod. Dev.* **68**, 441–448 (2004).
8. Graf, A. *et al.* Fine mapping of genome activation in bovine embryos by RNA sequencing. *PNAS* **111**, 4139–4144 (2014).
9. Tadros, W. & Lipshitz, H. D. The maternal-to-zygotic transition: a play in two acts. *Development* **136**, 3033–3042 (2009).
10. Cockburn, K. & Rossant, J. Making the blastocyst: lessons from the mouse. *J. Clin. Invest.* **120**, 995–1003 (2010).
11. Menchero, S., Rayon, T., Andreu, M. J. & Manzanares, M. Signaling pathways in mammalian preimplantation development: linking cellular phenotypes to lineage decisions. *Dev. Dyn.* **246**, 245–261 (2017).
12. Zhang, Y., Yang, Z. & Wu, J. Signaling pathways and preimplantation development of mammalian embryos. *FEBS J.* **274**, 4349–4359 (2007).
13. Plotnikov, A., Zehorai, E., Procaccia, S. & Seger, R. The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochim. Biophys. Acta Mol. Cell Res.* **1813**, 1619–1633 (2011).
14. Ashry, M. *et al.* Functional role of AKT signaling in bovine early embryonic development: potential link to embryotrophic actions of follistatin. *Reprod. Biol. Endocrinol.* **16**, 1 (2018).

15. Hu, J. *et al.* Vitamin C enhances the in vitro development of porcine pre-implantation embryos by reducing oxidative stress. *Reprod. Domest. Anim.* **47**, 873–879 (2012).
16. dos Santos, E. C. *et al.* The effects of crocetin supplementation on the blastocyst outcome, transcriptomic and metabolic profile of in vitro produced bovine embryos. *Theriogenology* **123**, 30–36 (2019).
17. Huang, H. *et al.* The multifunctional effects of nobiletin and its metabolites in vivo and in vitro. *Evid. Based Complement. Altern. Med.* **2016**, 1–14 (2016).
18. Cajas, Y. N. *et al.* Antioxidant nobiletin enhances oocyte maturation and subsequent embryo development and quality. *Int. J. Mol. Sci.* **21**, 5340 (2020).
19. Zhang, L. *et al.* Nobiletin promotes antioxidant and anti-inflammatory responses and elicits protection against ischemic stroke in vivo. *Brain Res.* **1636**, 130–141 (2016).
20. Ufer, C. & Wang, C. C. The roles of glutathione peroxidases during embryo development. *Front. Mol. Neurosci.* **4**, 12 (2011).
21. Mishra, A., Reddy, I., Gupta, P. & Mondal, S. L-carnitine mediated reduction in oxidative stress and alteration in transcript level of antioxidant enzymes in sheep embryos produced in vitro. *Reprod. Domest. Anim.* **51**, 311–321 (2016).
22. Lequarre, A. S., Marchandise, J., Moreau, B., Massip, A. & Donnay, I. Cell cycle duration at the time of maternal zygotic transition for in vitro produced bovine embryos: effect of oxygen tension and transcription inhibition. *Biol. Reprod.* **69**, 1707–1713 (2003).
23. Zullo, G. *et al.* Crocetin improves the quality of in vitro–produced bovine embryos: Implications for blastocyst development, cryotolerance, and apoptosis. *Theriogenology* **86**, 1879–1885 (2016).
24. Chowdhury, M. M. R. *et al.* Improved developmental competence in embryos treated with lycopene during in vitro culture system. *Mol. Reprod. Dev.* **85**, 46–61 (2018).
25. Ge, H. *et al.* The importance of mitochondrial metabolic activity and mitochondrial DNA replication during oocyte maturation in vitro on oocyte quality and subsequent embryo developmental competence. *Mol. Reprod. Dev.* **79**, 392–401 (2012).
26. Harvey, A. J. Mitochondria in early development: linking the microenvironment, metabolism and the epigenome. *Reproduction* **157**, R159–R179 (2019).
27. Jojua, N., Sharikadze, N., Zhuravliova, E., Zaalishvili, E. & Mikeladze, D. G. Nobiletin restores impaired hippocampal mitochondrial bioenergetics in hypothyroidism through activation of matrix substrate-level phosphorylation. *Nutr. Neurosci.* **18**, 225–231 (2015).
28. Dabrundashvili, N. G. *et al.* Nobiletin transiently increases the production of nitric oxide and changes the activity of succinate dehydrogenase in human blood lymphocytes. *J. Food Biochem.* **35**, 638–649 (2011).
29. Ghanem, N. *et al.* Differential expression of selected candidate genes in bovine embryos produced in vitro and cultured with chemicals modulating lipid metabolism. *Theriogenology* **82**, 238–250 (2014).
30. Wahli, W. & Michalik, L. PPARs at the crossroads of lipid signaling and inflammation. *Trends Endocrinol. Metab.* **23**, 351–363 (2012).
31. Childs, S. *et al.* Embryo yield and quality following dietary supplementation of beef heifers with n-3 polyunsaturated fatty acids (PUFA). *Theriogenology* **70**, 992–1003 (2008).
32. Mulvihill, E. E. *et al.* Nobiletin attenuates VLDL overproduction, dyslipidemia, and atherosclerosis in mice with diet-induced insulin resistance. *Diabetes* **60**, 1446–1457 (2011).
33. Lin, Y. *et al.* Molecular structures of citrus flavonoids determine their effects on lipid metabolism in HepG2 cells by primarily suppressing ApoB secretion. *J. Agric. Food Chem.* **59**, 4496–4503 (2011).
34. Canovas, S., Ross, P. J., Kelsey, G. & Coy, P. DNA methylation in embryo development: epigenetic impact of ART (assisted reproductive technologies). *BioEssays* **39**, 1700106 (2017).
35. Ramos-Ibeas, P. *et al.* Embryo responses to stress induced by assisted reproductive technologies. *Mol. Reprod. Dev.* **86**, 1292–1306 (2019).
36. Cebrian-Serrano, A. *et al.* Effect of the bovine oviductal fluid on in vitro fertilization, development and gene expression of in vitro-produced bovine blastocysts. *Reprod. Domest. Anim.* **48**, 331–338 (2013).
37. He, L. *et al.* Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. *CPB* **44**, 532–553 (2017).
38. Zou, W. *et al.* PI3K/Akt pathway mediates Nrf2/ARE activation in human L02 hepatocytes exposed to low-concentration HBCDs. *Environ. Sci. Technol.* **47**, 12434–12440 (2013).
39. Harris, C. & Hansen, J. M. Oxidative Stress, Thiols, and Redox Profiles. in *Developmental Toxicology: Methods and Protocols* (eds. Harris, C. & Hansen, J. M.) 325–346 (Humana Press, 2012). https://doi.org/10.1007/978-1-61779-867-2_21.
40. Wu, X. *et al.* Nobiletin and its colonic metabolites suppress colitis-associated colon carcinogenesis by down-regulating iNOS, inducing antioxidative enzymes and arresting cell cycle progression. *J. Nutr. Biochem.* **42**, 17–25 (2017).
41. Liu, P. *et al.* Cell-cycle-regulated activation of Akt kinase by phosphorylation at its carboxyl terminus. *Nature* **508**, 541–545 (2014).
42. Milazzotto, M. P. *et al.* The mechanism of oocyte activation influences the cell cycle-related genes expression during bovine pre-implantation development. *Cell. Reprogram.* **14**, 418–424 (2012).
43. Lien, L.-M. *et al.* Nobiletin, a polymethoxylated flavone, inhibits glioma cell growth and migration via arresting cell cycle and suppressing MAPK and Akt pathways. *Phytother. Res.* **30**, 214–221 (2016).
44. Jang, C.-W., Shibata, Y., Starmer, J., Yee, D. & Magnuson, T. Histone H3.3 maintains genome integrity during mammalian development. *Genes Dev.* **29**, 1377–1392 (2015).
45. Zhang, K., Wang, H., Rajput, S. K., Folger, J. K. & Smith, G. W. Characterization of H3.3 and HIRA expression and function in bovine early embryos. *Mol. Reprod. Dev.* **85**, 106–116 (2018).
46. Wen, D. *et al.* Genome editing a mouse locus encoding a variant histone, H3.3B, to report on its expression in live animals: genome editing of the endogenous H3.3B ALLELE. *Genesis* **52**, 959–966 (2014).
47. Lee, M. T. *et al.* Nanog, Pou5f1 and SoxB1 activate zygotic gene expression during the maternal-to-zygotic transition. *Nature* **503**, 360–364 (2013).
48. Lindeman, L. C. *et al.* Prepatterning of developmental gene expression by modified histones before zygotic genome activation. *Dev. Cell* **21**, 993–1004 (2011).
49. Sivanand, S., Viney, I. & Wellen, K. E. Spatiotemporal control of Acetyl-CoA metabolism in chromatin regulation. *Trends Biochem. Sci.* **43**, 61–74 (2018).
50. Zhou, W., Nie, Z.-W., Zhou, D.-J. & Cui, X.-S. Acetyl-CoA synthases are essential for maintaining histone acetylation under metabolic stress during zygotic genome activation in pigs. *J. Cell. Physiol.* https://doi.org/10.1002/jcp.30355 (2021).
51. Manning, B. D. & Toker, A. AKT/PKB signaling: navigating the network. *Cell* **169**, 381–405 (2017).
52. Marques, L. & Thorsteinsdóttir, S. Dynamics of Akt activation during mouse embryo development: distinct subcellular patterns distinguish proliferating versus differentiating cells. *Differentiation* **86**, 48–56 (2013).
53. Dummler, B. *et al.* Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. *Mol. Cell Biol.* **26**, 8042–8051 (2006).
54. O'Neill, C. Phosphatidylinositol 3-kinase signaling in mammalian preimplantation embryo development. *Reproduction* **136**, 147–156 (2008).
55. Rizos, D., Ward, F., Duffy, P., Boland, M. P. & Lonergan, P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* **61**, 234–248 (2002).
56. Lopera-Vasquez, R. *et al.* Effect of bovine oviductal fluid on development and quality of bovine embryos produced in vitro. *Reprod. Fertil. Dev.* **29**, 621–629 (2017).

57. Holm, P., Booth, P. J., Schmidt, M. H., Greve, T. & Callesen, H. High bovine blastocyst development in a static in vitro production system using sofaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology* **52**, 683–700 (1999).
58. del Collado, M. *et al.* Influence of bovine serum albumin and fetal bovine serum supplementation during in vitro maturation on lipid and mitochondrial behaviour in oocytes and lipid accumulation in bovine embryos. *Reprod. Fertil. Dev.* **28**, 1721–1732 (2015).
59. Bermejo-Álvarez, P., Rizos, D., Rath, D., Lonergan, P. & Gutierrez-Adan, A. Epigenetic differences between male and female bovine blastocysts produced in vitro. *Physiol. Genom.* **32**, 264–272 (2008).
60. Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P. & Gutierrez-Adan, A. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *PNAS* **107**, 3394–3399 (2010).
61. Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative C T method. *Nat. Protoc.* **3**, 1101–1108 (2008).
62. Hamdi, M. *et al.* Gene expression and metabolic response of bovine oviduct epithelial cells to the early embryo. *Reproduction* **158**, 85–94 (2019).
63. Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, research0034.1 (2002).
64. Sánchez-Calabuig, M. J. *et al.* A high glucose concentration during early stages of in vitro equine embryo development alters expression of genes involved in glucose metabolism. *Equine Vet. J.* https://doi.org/10.1111/evj.13342 (2020).
65. López-Cardona, A. P. *et al.* Exocannabinoids effect on in vitro bovine oocyte maturation via activation of AKT and ERK1/2. *Reproduction* **152**, 603–612 (2016).
66. Ashry, M. *et al.* Expression of TGFβ superfamily components and other markers of oocyte quality in oocytes selected by brilliant cresyl blue staining: Relevance to early embryonic development. *Mol. Reprod. Dev.* **82**, 251–264 (2015).

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Author contributions

K.C.-B., Y.N.C., D.R. and E.M.G. conceived and designed the study. K.C.-B., Y.N.C., S.P.-C., C.L.V.L. and E.M.G. performed the experiments. K.C.-B., Y.N.C., E.A. and D.R. analysed and interpreted the data. K.C.-B. and Y.N.C. wrote the first draft of the manuscript. C.L.V.L., E.A., A.G.-A., E.M.G. and D.R. revised and discussed the manuscript. A.G.-A. and D.R. Funding acquisition. All authors read and approved the manuscript for publication.

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Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to D.R.

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Biology of Reproduction

Nobiletin-induced partial abrogation of deleterious effects of AKT inhibition on preimplantation bovine embryo development in vitro

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Corresponding Author:	Dimitrios Rizos, PhD INIA: Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria Madrid, Madrid SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	INIA: Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria
Corresponding Author's Secondary Institution:	
First Author:	Yulia N Cajas
First Author Secondary Information:	
Order of Authors:	Yulia N Cajas
	Karina Cañón-Beltrán, PhD
	Carolina Núñez-Puente
	Alfonso Gutierrez-Adán, PhD
	Encina M González, PhD
	Ekaitz Agirregoitia, PhD
	Dimitrios Rizos, PhD
Order of Authors Secondary Information:	
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Abstract:	During preimplantational embryo development, PI3K/AKT regulate cell proliferation and differentiation and nobiletin modulate this pathway to promote cell survival. Therefore, we aimed to establish whether, when the AKT cascade is inhibited using inhibitors III and IV, nobiletin supplementation to in vitro culture media during the minor (2 to 8-cell stage, MNEGA) or major (8 to 16-cell stage, MJEGA) phases of EGA is able to modulate the development and quality of bovine embryos. In vitro zygotes were cultured during MNEGA or MJEGA phase in SOF+5% FCS or supplemented with: 15μM AKT-InhIII; 10μM AKT-InhIV; 10μM nobiletin; nobiletin+AKT-InhIII; nobiletin+AKT-InhIV; 0.03% DMSO. Embryo development was significantly lower in treatments with AKT inhibitors, while combination of nobiletin with AKT inhibitors was able to recover their adverse developmental effect and also increase blastocyst cell number. The mRNA abundance of GPX1, NFE2L2, and POU5F1 was partially increased in 8- and 16-cell embryos from nobiletin with AKT inhibitors. Besides, nobiletin increased the p-rpS6 level whether or not AKT inhibitors were present. In conclusion, nobiletin promotes bovine embryo development and quality and partially recover the adverse developmental effect of AKT inhibitors which infers that nobiletin probably uses another signaling cascade that PI3K/AKT during early embryo development in bovine.

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