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- Partially evaluated by novel fluorescent staining

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Effect of insulin during oocyte maturation *in vitro* on bovine early embryo development – Partially evaluated by novel fluorescent staining

Påverkan av insulin vid oocytmognad *in vitro* under tidig embryoutveckling hos nöt – Delvis utvärderat med hjälp av en ny fluorescensfärgning

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SUMMARY

Fertility is of central interest in the dairy production but has during the last decades declined. Increased milk yield has resulted in high pressure on the metabolism of the dairy cows that are supposed to manage the transition from dry cows to lactating cows within a few weeks around the parturition. Much indicate that metabolism and fertility are closely linked, with insulin playing a substantial part. There are many studies suggesting that the main part of gestation loss can be found during the early embryo development, a period which can be studied *in vitro*. The aim of this study was to test the effect of insulin during maturation *in vitro* and to evaluate two different fluorescent stainings on oocytes and embryos; a nuclear stain and a staining of apoptotic cells through the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-method.

Bovine cumulus-oocyte-complexes (*n=991)* were aspirated from abattoir-derived ovaries. The oocytes were matured *in vitro* under different insulin-conditions (Z: 0 µg/ml L: 0.1 µg/ml H: 10 µg/ml). After maturation, oocytes were either removed for staining or fertilized and cultured *in vitro* to day eight after fertilization. All blastocysts were morphologically assessed at day seven and eight. Cumulus-oocyte-complexes removed before fertilization and day eight blastocysts were later stained with TUNEL- and nuclear stain for evaluation. Statistical analysis was executed on arc sinus squareroot transformed values. The effect of treatments were analysed by ANOVA.

132 matured oocytes and 230 day eight blastocysts from eight batches were analysed. There was no significant difference between the treatments regarding oocytes cleaved 44h post fertilization (Z:0.85±0.02 L: 0.85±0.02 H: 0.89±0.03, $p>0.05$). Insulin tended to impair the fraction of blastocysts developed on day seven from cleaved oocytes (Z: 0.22±0.02 L: 0.17±0.02 H: 0.14±0.02, $p=0.05$). There was a tendency for insulin treated groups to have more of the earlier development stages and also less blastocysts of high quality grades when morphologically assessed at day eight compared to control group. The difference was significant when comparing treated groups together versus the control group ($p<0.05$). There was no significant difference in apoptotic cells in the blastocysts (Z: 0.021±0.003 L: 0.026±0.003 H: 0.028±0.003) nor in apoptotic cells in the outer two layers of the cumulus-complexes (Z: 0.052±0.025 L: 0.039±0.016 H: 0.077±0.044).

In this study, insulin during oocyte maturation *in vitro* impaired the blastocyst development both regarding fraction, stage and grade at day seven and eight. The TUNEL-kit evaluated did not work satisfactory for staining of embryos and cumulus-oocyte-complexes with multiple cell-layers. The nuclear staining tested however, seem to be a good candidate for staining of blastocysts and cumulus-oocyte-complexes with a limited amount of cell-layers. The stain is easy to use and further did not bleach after several weeks of storage.

SAMMANFATTNING

Fertiliteten är essentiell i mjölkproduktionen men har under de sista decennierna minskat. Den ökade mjölkproduktionen sätter hög press på mjölkkons metabolism då hon förväntas ställa om från sinko till högmjölkande ko på ett fåtal veckor kring kalvning. Mycket pekar på att anledningen till den nedsatta fertiliteten hos mjölkkorna härör från metabolismen, där insulin har en betydande roll. Många studier visar också på hur majoriteten av dräktighetsförlusterna sker under den tidiga embryoutvecklingen, vilket är en period som kan simuleras *in vitro,* det vill säga i laboratoriemiljö. Syftet med denna studie var att testa insulinets påverkan på mognad av äggceller *in vitro* samt att utvärdera en metod för infärgning av kärnor och en annan metod för apoptotiska celler i äggceller och tidiga embryon.

Äggceller omgivna av stödceller *(n=991)* från nötkreatur samlades från äggstockar hämtade från slakteri. Äggcellerna fick mogna i tre olika insulinkoncentrationer *in vitro* (Z: 0 µg/ml L: 0,1 µg/ml H: 10 µg/ml). Efter mognad *in vitro*, avlägsnades antingen äggcellerna för färgning eller fick fortsätta *in vitro* produktionen genom befruktning och odling upp till dag åtta efter befruktningen. Äggcellkomplex som avlägnats före befruktningen samt blastocyster (tidiga embryon) från dag åtta färgades därefter med en kärnfärgning och en TUNEL-färgning för att detektera apoptos. Statistisk analys av resultaten utfördes av ANOVA, med arc sinus kvadratrot-transformerade värden.

Mognade äggcellskomplex (*n=132*) och blastocyster *(n=230)* från åtta omgångar ingick i studien. Det var ingen signifikant skillnad mellan de olika grupperna avseende äggceller som delat sig 44 timmar efter befruktningen (Z:0,85±0,02 L: 0,85±0,02 H: 0,89±0,03, $p>0,05$). Insulin tenderade att ha en negativ effekt på blastocystutvecklingen, med signifikanta värden vid dag sju-blastocyster räknat från antalet äggceller som delat sig 44 timmar efter befruktningen (Z: 0,22±0,02 L: 0,17±0,02 H: 0,14±0,02, $p=0,05$).

Det fanns en tendens att insulin även bromsade utvecklingen och försämrade kvaliteten av bildade blastocyster: Denna skillnad var signifikant vid jämförelse mellan insulin-behandlade grupper mot kontrollgruppen ($p<0,05$). Ingen skillnad kunde ses på andelen apoptotiska celler i blastocyster (Z: 0,021±0,003 L: 0,026±0,003 H: 0,028±0,003) eller i apoptotiska celler i de yttersta två lagren av cumuluskomplexen (Z: 0,052±0,025 L: 0,039±0,016 H: 0,077±0,044).

Denna studie visar hur insulin under äggcellens mognad *in vitro* påverkar embryoutvecklingen negativt, något som överensstämmer med tidigare studier. Tidigare studier har dock till viss del visat motsatta resultat avseende den negativa effekt av insulin som kunde ses på utvecklingsstadie och kvalitet i denna studie. TUNEL-färgningen som utvärderades i studien hade inte tillräckliga egenskaper för användning på embryon och äggcellskomplex, däremot visade kärnfärgningen goda egenskaper vilken gör den tillfredsställande i användning på embryon och äggcellskomplex.

CONTENT

ABBREVIATIONS

*from cleaved	Fraction from cleaved oocytes assessed at 44h post fertilization
*from fertilized	Fraction from fertilized presumed zygotes transferred to culture
AI	Apoptotic index (Fraction apoptotic cells)
BSA	Bovine Serum Albumin
CA2	Fraction oocytes cleaved above two cells assessed 44h post fertilization
CC	Cumulus cells
Cleaved	Fraction cleaved oocytes assessed 44h post fertilization
CO_2	Carbon dioxide
COCs	Cumulus oocyte complexes
D7B	Day seven blastocysts
D8B	Day eight blastocysts
dsDNA	Double stranded deoxyribonucleic acid
dUTP	Deoxyurine triphosphate
FSH	Follicle stimulating hormone
H	Group treated with 10 µg/ml insulin during maturation *in vitro* (high)
Hoechst	Hoechst 33342, 2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole
ICM	Inner cell mass
IGF-1	Insulin-like growth factor type 1
IVF	*In vitro* embryo fertilization
IVM	*In vitro* embryo maturation
IVP	*In vitro* embryo production
L	Group treated with 0.1 µg/ml insulin during maturation *in vitro* (low)
LH	Luteinizing hormone
M	Molar
mSOF	Modified synthetic oviductal fluid

NEB	Negative energy balance
N_2	Nitrogen
ng	Nanogram
nM	Nanomolar
ns	Not significant
O_2	Oxygen
PEB	Positive energy balance
PFA	Paraformaldehyde
PVA	Polyvinylalcohol
RT	Room temperature
Tb	Trophoblasts
TdT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
w/v	Weight to volume
Z	Control group, not treated with insulin during maturation *in vitro* (zero)
µg	Microgram
µl	Microlitre
µM	Micromolar

INTRODUCTION

Fertility is of central interest in the dairy production as the birth of a new calf with even intervals is essential for milk-production. Fertility has declined during the last decades and some indicate that the early embryo development is a reason for the decline. As the high metabolism of the dairy cow results in great strain on the metabolic apparatus the effect of different metabolic parameters on fertility is of interest. One model for studying the early embryo development is using the *in vitro* production of embryos and the subsequent evaluation of embryos produced.

The aim of this study was to test the effect of insulin during maturation of bovine oocytes *in vitro*. Embryos were evaluated through assessment of morphological development and further through staining with a nuclear and apoptotic stain also evaluated in the study.

LITERATURE REVIEW

Fertility in dairy cows

In the dairy production, the fertility is of central interest as the birth of a calf with even intervals is essential for milk production (Leroy *et al.* 2008a). Reduced fertility results in prolonged calving intervals, increased age at first calving, decreased production and the eventually higher expenses of veterinary care, all which causes economic losses for the farmer (Sveriges Veterinärmedicinska Anstalt, 2012).

The fertility has declined during the last decades as the milk yield has increased (Butler & Smith 1989; Gong *et al.* 2002; Leroy *et al.* 2008b). The oocytes have been shown to be of lower quality in the high yielding cow than in the correspondent cow with low yield (Butler & Smith 1989; Leroy *et al.* 2008b, Snijders *et al.,* 2000) and the oocytes of traditionally used Holstein breed (with marginally higher yield than the Swedish red breed) have been shown to have less competence to develop into blastocysts *in vitro* than those of the Swedish red breed (Abraham *et al.* 2012).

The increased milk yield results in increased metabolism which puts great strain on the metabolic system of the dairy cow. The transition period (the first week's pre partum to the weeks post partum) is the most challenging metabolic period where the cow is supposed to manage the transition from the dry period (positive energy balance, PEB) to the early lactating period (negative energy balance, NEB). During this time, the organism has to adapt to different (and even opposing) metabolic conditions (Gordon 2003, pp.103–104). As the final development of an oocyte before ovulation takes approximately 8-12 weeks, oocytes ready for fertilization at the first insemination after calving will be subjected to the metabolic stress of PEB/NEB the cow is being subjected to (Beam & Butler 1997; Gordon 2003, pp.65–66; Leroy *et al.* 2008a). The energy status in mid-lactation when the cow does not suffer from NEB generates oocytes with increased quality compared to oocytes ovulated during the early lactation (Gordon 2003, p.103). Further, diets reducing the time in NEB (starch-rich or fat-rich diets) increase the quality of oocytes produced early post partum (Beam & Butler 1997; Gong *et al.* 2002).

Diet does affect the oocyte quality and diet increasing growth rate during oocyte maturation decreases both blastocyst formation and quality (Freret *et al,* 2006). Overweigh impair blastocyst formation and further, overfeeding in already overweight heifer seem to intensify the negative effect (Awasthi *et al.,* 2010). Though, following a period of overfeeding, a restricted diet can be beneficial for the early embryo development (Freret *et al.,* 2006).

Bovine early embryo development

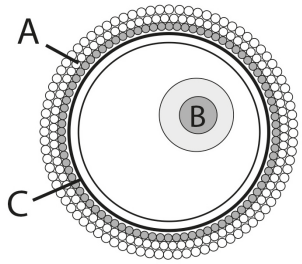

Figure 1. *Cumulus-oocyte-complex (COC).* Oocyte with polar body (B), surrounded by a zona pellucida (C) and granulosa cells forming the cumulus complex (A). Illustration: Johan Hallberg

The development of an embryo starts with the oocyte maturing in the female gonad, the ovary. Matured oocytes are formed from primordial follicles developing during fetal growth to primary follicles which are preserved in the mammalian ovary. During several estrous cycles follicles develop from primary follicles to secondary and maturing, or antral follicles, a process which takes approximately 8-12 weeks. In these stages, the oocyte is enveloped and nourished by layers of granulosa cells and the the follicle in turn is surrounded by theca cells participating in the production of estradiol from the follicle. The granulosa-cells together with the oocyte, produce the zona pellucida, a thick layer of glycoproteins covering the oocyte (Beam & Butler 1997; Sjaastad *et al.* 2003, pp.640–645; Gordon 2003, pp.65–66).

During the maturation of the oocyte the granulosa cells develop into two different strains of cells, epithelial mural granulosa cells which stay in contact with the basal membrane of the follicle and cumulus cells (CCs), enclosing the oocyte and initiate contact between oocyte and surrounding mural granulosa cells (Gordon 2003, pp.64–65). The connection between the oocyte and the granulosa cells are formed by gap junctions, which functions is mainly to provide the oocyte with nutrition and regulatory proteins, but also have a role in the LH-dependent maturation of the oocyte (Adamiak 2005).

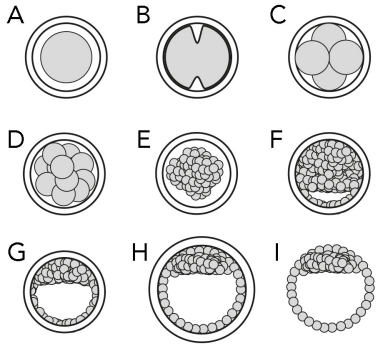

Figure 2: *Early embryo development.* A: Oocyte. B: Cleaving oocyte, C-D: 4-16 cell stage embryo (Day 3-5), E: Morula (Day 5-6), F: Early blastocyst (Day 7), G: Blastocyst (Day 7-8), H: Expanding blastocyst (Day 8-9), I: Hatched blastocyst (Day 9). Modified from IETS Manual, 2010. Illustration: Johan Hallberg

Initiated by a LH-peak in plasma, fluid is accumulated in the maturing follicles, contractions of smooth muscle cells in the ovaries initiates and blood flow to the ovary is increased.

These actions together initiate the ovulation. The ovulated oocyte is still covered by layers of sticky granulosa-cells, together with the oocyte forming the cumulus oocyte complex (COC) (Sjaastad *et al.* 2003, p.645) *(Figure 1).*

Fertilization takes place in the oviduct, to which sperm have been transported through the female reproductive tract and undergone the capacitation process which prepare the spermatozoa for fertilization. A spermatozoon is binding and penetrating the zona pellucida resulting in fertilization;

the transfer of the spermatozoon's nucleus to the oocyte (Sjaastad *et al.* 2003, pp.654–655).

The fertilized oocyte, the zygote, starts to divide as soon as it is formed. Initially, division is completed once every 24h. The early embryo forms through cell division to an undifferentiated cell mass, morula, at 5-7 days post fertilization (Betteridge & Flechon 1988). One or two days later, the morula develop to a blastocyst where the blastomeres have differentiated into trophoblasts (Tb) surrounding the blastocoele, later forming the fetal membranes, and an inner cell mass (ICM), a compact cell cluster which will start to form the embryo. The zona pellucida still protects the blastocyst until day 7-10 post fertilization when the zona pellucida is weakened and ruptures and the blastocyst hatches (Betteridge & Flechon 1988; Sjaastad *et al*. 2003, pp.656–657) *(Figure 2)*.

In vitro model for oocyte and embryo development

In vitro production (IVP) of embryos is a production of embryos within a laboratory where the *in vivo* situation are mimicked during the first week of embryo development. As the use of live animals should be minimized, IVP gives the opportunity to study the oocyte maturation and the early embryo development of the cow without the use of experimental animals. IVP is also less expensive than animal-trials and gives the oppurtunity to use a large amount of oocytes for the study, increasing the viability of the results of the hypothesis tested (Leroy *et al.* 2008b).

In vitro production of bovine embryos

The outline of IVP of bovine embryos differs between protocols but is in most respects the same. Oocytes are collected through COCs which are aspirated, matured, fertilized and cultured *in vitro* and are thereafter analysed in different manners to test the a work hypothesis.

COCs are collected either through aspiration from ovaries collected from the slaughterhouse or from live cattle *e.g.* through ovum-pick-up techniques. COCs are after this selected on given standards and incubated in humidified CO_2-chamber for maturation *in vitro*. The CO_2-chamber has constant temperature for optimal culture enviroment for culture and standardized trials. The CO_2 and O_2 levels are modulated to simulate the *in vivo* situation, with higher CO_2 pressure and lower O_2 pressure than the atmosphere to prevent the production of free radicals. The chamber is humified to avoid media from evaporating. After maturation, the *in vitro* maturated oocytes are prepared

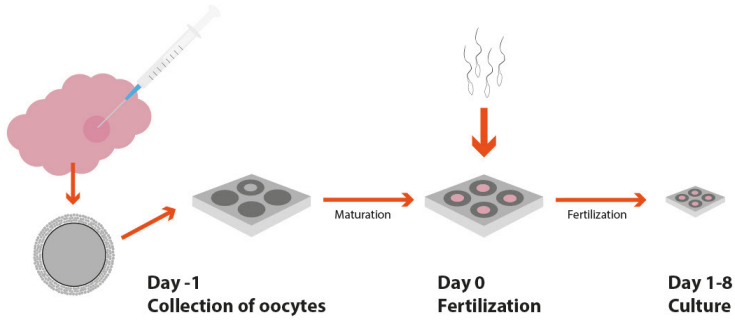

Figure 3: *Overwiev of in vitro production of embryos.* COCs are collected and incubated for maturation at day -1. At day 0 the oocytes are fertilized and further cultured for 8 days. Illustration: Johan Hallberg

for fertilization and sperm (usually frozen semen) are added to the oocytes at a given concentration for fertilization *in vitro* during incubation. After fertilization, the oocytes are transferred to culture media for further development and raised to day seven (D7B) or eight blastocysts (D8B) *(Figure 3)*.

The only certain method to draw conclusions about the quality of an oocyte/embryo produced *in vitro* is the transfer to a recipient to prove its potential to result in a living calf, but as this is neither practically or ethically possible in all situations, other evaluation methods of oocytes and embryos are necessary (Leroy *et al.* 2008a). The methods chosen for evaluation should be well correlated with the quality of the oocyte/embryo and preferably be non-invasive as this enables later embryo transfer (ET). Well established and broadly used non-invasive methods to assess blastocyst development are among others time of cleavage of the zygote and morphology and rate of blastocysts formed. Time of cleavage is assessed at given time post fertilization and regard is taken to whether the oocyte has cleaved once (Cleaved) or above 2 cell stage (CA2). Blastocyst development is assessed by light-microscopic inspection on D7B and D8B regarding developmental stage and quality grade.

Insulin

Insulin is a peptide hormone produced by the β-cells in the islets of Langerhans in the pancreas. Insulin is an important anabolic hormone and in cattle, the secretion is stimulated after food-intake by enhanced free fatty acid (FAF)- and amino-acid-concentrations in the blood. Increased food intake increases the levels of insulin in the blood (Freret *et al.,* 2006), as does starch-rich diets (Gong *et al.* 2002; Garnsworthy *et al.* 2009). Insulin stimulates uptake of nutrients in the cells and also stimulate synthesis of proteins and triglycerides (Sjaastad *et al.* 2003).

Insulin levels in plasma depends on the follicular/luteal phase and varies up from approximately 0.1-10 ng/ml (Spicer & Echternkamp 1995; Adamiak *et al.* 2005; Garnsworthy *et al.* 2009). Most metabolites have the same or lower concentration in follicular fluid compared to plasma due to the production of follicular fluid being a result of filtering of the plasma. Levels measured in follicular fluid are 0.1-10 ng/ml (Spicer & Echternkamp 1995; Landau *et al.* 2000). Insulin levels in follicular fluid conform with plasma levels indicating that nutritional status affect the systemic and hence the ovarian levels of insulin (Spicer & Echternkamp 1995).

Insulin is decreased during the first weeks postpartum, *i.e.* during the period of NEB and is thereafter increasing during the following 2-7 weeks making insulin a good marker for the control whether the cow has adapted her metabolism/managed to overcome the NEB (Leroy *et al.* 2008b; Garnsworthy *et al.* 2009).

Insulin and fertility

Many studies has shown links between fertility and insulin (Spicer & Echternkamp 1995; Matsui *et al.* 1995a; Beam & Butler 1997; Bowles & Lishman 1998; Landau *et al.* 2000;

Gong *et al.* 2002; Adamiak *et al.* 2005; Garnsworthy *et al.* 2009). As in other cells, insulin have a stimulatory effect on the ovary (Leroy *et al.* 2008b) and appears to mediate the effect on cells through the Type I IGF receptor . Insulin stimulates the proliferation of granulosa-cells, the DNA synthesis and the stereoidogenesis in theca cells enhancing the progesterone production. Physiological levels of insulin (<10 ng/ml) stimulate the follicular estradiol production (Spicer & Echternkamp 1995) and in mice, it has been shown that insulin stimulate the DNA, RNA and protein synthesis from morula-stage (Rao *et al.* 1990). As an important regulatory hormone of the metabolism, deviation from physiological levels can harm fertility and the effect of insulin on early embryos and oocytes may differ as the requirements of the environment differs between the maturing oocyte and the early embryo development. The effect of insulin on early embryo development is also different between species.

Body condition does affect the fertility (Snijders *et al.,* 2000) and deviation of the physiological level of insulin, through elevated food intake or obesity, can lead to fertility problems. Hyperinsulinemiae is linked to decreased oocyte-quality *in vivo* as Adamiak *et al* (2005) showed in a study comparing insulinemic (starch-rich) diets with control groups in cows. On the other hand, Beam & Butler (1997) also showed how diets with higher fat content decrease the duration of low insulin condition, which consequences in increased diameter of follicles postpartum.

Effect on oocyte maturation

Landau *et al* (2000) suggested that insulin has impact on oocyte-maturation *in vivo* as they saw increased levels of insulin in preovulatory follicles compared to subordinate follicles. Insulin in supra-physiological concentrations has shown to have a stimulatory effect on cleavage and maturation of oocytes *in vitro* (Bowles & Lishman 1998; Gong *et al.* 2002). This agrees with the suggestion that insulin affects the follicular development *in vivo* and affects the beginning of follicular development early post partum (Bowles & Lishman 1998; Landau *et al.* 2000; Gong *et al.* 2002; Garnsworthy *et al.* 2009).

Effect on early embryo development

Many studies have shown that insulin seems to have effect on early embryo development *in vitro*. However, the results are not evident and even contradictory, showing both increased development at morula stage *in vitro* (Matsui et al. 1995a) and decreased development to blastocyst stage *in vitro* (Bowles & Lishman 1998). The consequences for the embryo created are difficult to evaluate and further tests on long-term *in vivo* effects are poorly understood.

Bowles and Lishman (1998) showed how insulin affected early embryo development *in vitro* by increasing the amount of blastomeres. They suggested that this is the reason for media containing insulin need addition of glucose and/or amino acid for showing the positive effect of insulin on embryo development (Bowles & Lishman 1998).

As insulin has been shown to have a positive effect on bovine blastocyst rate in different studies, there are protocols of *in vitro* production of embryos using insulin in the culture medium (Choi *et al.* 1998).

Evaluation of oocytes and embryos

As is it not always possible to transfer the embryos created *in vitro* to a living recipient, other techniques for evaluating embryos are necessary. Unfortunately, the optimal test is yet to be found and still we stand with mostly invasive methods of evaluating embryos and oocytes.

Among the methods following, are some examples of tests commonly used today for evaluating oocyte and/or embryo quality; *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and IVP. Further diagnostic evaluations as apoptotic index (AI), morphology of oocyte, morphology of COCs and embryo, genomic transcripts, nucleus stage and lipid content (Leroy *et al.* 2008b). The meiotic stage of the nucleus is assessed though visualisation of a polar body on oocytes developing to M2-stage.

Apoptosis

Apoptosis is crucial to cell development in multicellular organisms and different types of cell death (apoptosis, necrosis, degeneration) can be found in normal embryo development.

Necrosis occurs from injury, due to damage on neighboring cells by triggering the inflammatory response. Apoptosis on the other hand is a highly conserved process, and is triggered within the cell through advanced signaling paths resulting in DNA-fragmentation (Gavrieli *et al.* 1992). As apoptosis and necrosis are executed through different paths the morphology of the processes is also different. The morphological appearance of apoptosis include chromatin condensation and compactness of cytoplasmic organelles (Gavrieli *et al*. 1992). Soon after the apoptotic process, the remains of the cell are phagocytized (Gavrieli *et al.* 1992). Apoptosis can be used to evaluate the quality in oocytes and embryos (Roth & Hansen 2004; Rodríguez *et al.* 2006).

Visualizing apoptosis

TUNEL-stain

The terminal deoxynucleotide transferase-dUTP nick end labeling (TUNEL) assay enables *in situ*–apoptosis detection by incorporating modified dUTPs by the enzyme terminal deoxynucleotide transferase (TdT) to the ultimate terminator of apoptosis; the 3'end of the fragmented DNA. The dUTP is modified with a fluorophore, *i.e.* fluoreschine-dUTP, which is detectable directly with fluorescent microscopy or indirectly with antibodies attached to the molecule (Negoescu *et al.* 1996) *(Figure 4)*.

The TUNEL-method is the method of choice for identification of apoptotic cells in a population, however, the penetration through cell cultures depends on conditions regarding cell types and permeabilization (Negoescu *et al.* 1996).

The Click-it TUNEL assay has been used and published on cell cultures (Wang et al. 2012) before but as far as known not yet tested on embryos and oocytes.

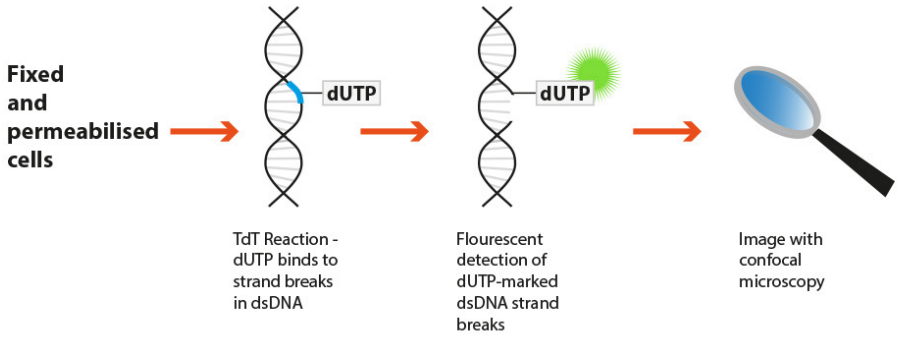

Figure 4. *Apoptotic visualisation by TUNEL-staining.* Fixed and permeabilised cells are run through a reaction with terminal deoxyribonucleotide transferase-dUTP which enables marking of dsDNA showing apoptotic strand-brakes. The dUTP ends are modified with a fluorophore making the reaction visible through fluorescent microscopy. Modified from Click-it TUNEL assay manual, 2008. Illustration: Johan Hallberg

Nuclear stain

A nuclear marker with fluorescence spectrum distinguished from other stainings is necessary for evaluating blastocysts and oocyte nuclear stage through confocal imaging.

The nuclear staining Hoechst 33342 is commonly used on oocytes and embryos (Makarevich & Markkula 2002; Martin *et al.* 2005; Rodríguez *et al.* 2006), however the confocal microscope used in this study (Zeiss LSM 510) cannot excite Hoechst why another nuclear stain was necessary. The nuclear staining used in this study was DraQ-5. The DraQ-5 staining is a fluorochrome developed for this purpose, excitation and emission on 633/647 nm (Smith *et al.* 2000) which makes the stain ideal for use on Zeiss LSM 510. Hoechst was used as counterstaining in this study to evaluate the specifics of the DraQ-5.

Aim of the study

In this study the effect of insulin during maturation was tested on early embryo development *in vitro* by adding insulin in the maturation medium at two different levels compared to a control group with no insulin added. The embryo development was follow until day eight post fertilization and evaluated in different manners.

Further, a new TUNEL-method was evaluated regarding whether or not it could be used on bovine COCs and embryos. A nuclear stain was evaluated parallel to this, assessing the compatibility with the TUNEL-stain for use on bovine COCs and embryos.

MATERIAL AND METHODS

Experimental design

Abattoir-derived bovine ovaries independent of breed or age were used. After transportation to the laboratory, COCs were aspirated followed by *in vitro* maturation, fertilization and culture according to standard protocols. The oocytes were divided into three treatment groups and matured under three different insulin-conditions: high (H) with supplement of 10 µg/ml bovine insulin, low (L) with supplement of 0.1 µg/ml insulin or control group (Z) with no supplement of insulin. Eight batches were run during seven weeks (October-November 2013). Each batch contained three groups with 33-48 COCs and a total of *n=991* COCs were included for maturation *in vitro* in all batches. After maturation, 4-12 oocytes per group were removed and fixed in paraformaldehyde (PFA) for staining. Remaining oocytes were fertilized with semen from one single diary breed bull with proven field and *in vitro* fertility. Embryo development was followed by recording cleavage rate 44h post fertilization and development and morphology was assessed on day seven and eight. On day eight, the morphological quality of blastocysts was recorded and blastocysts were fixed in PFA. Embryos and maturated oocytes were stored in phosphate buffered saline (PBS) containing polyvinylalcohol (PVA) before staining. Stainings used was Click-it TUNEL assay for apoptosis and DraQ-5 for nuclear staining. Laser scanning confocal microscope Zeiss LSM 510 (Zeiss, Oberkochen, Germany) was used for analysis of oocytes and embryos.

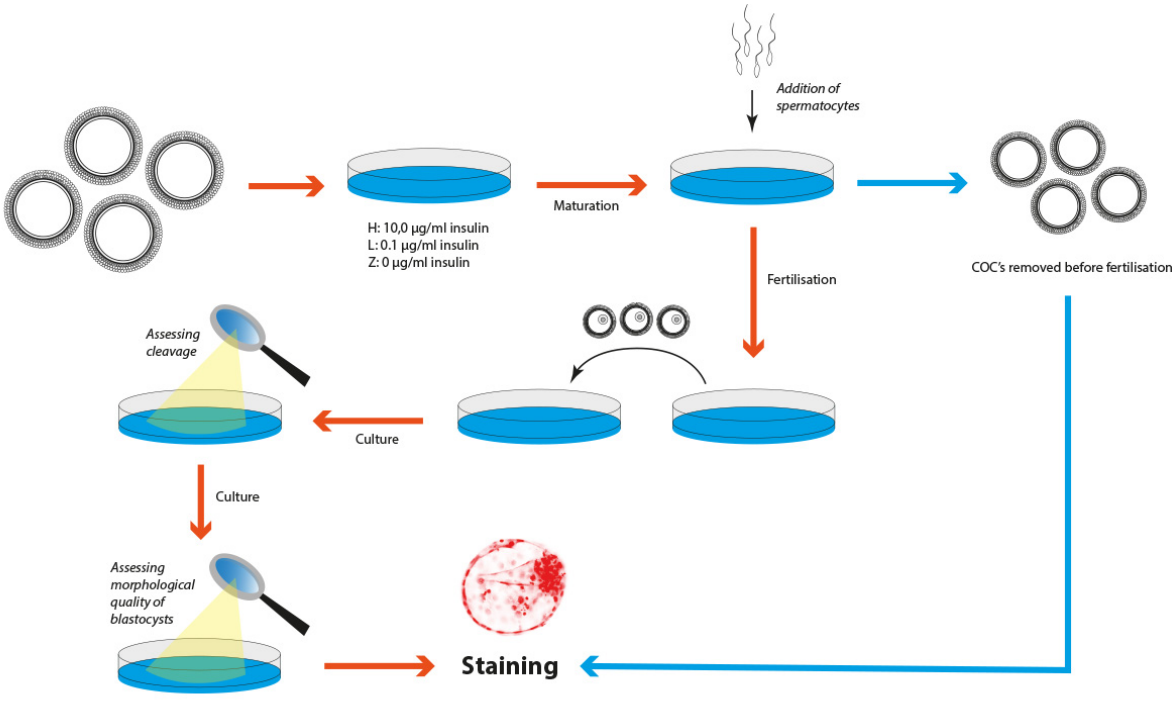

Figure 5. *Outline of experiment in the study.* COCs were collected and matured *in vitro* under different insulin conditions. COCs were removed after fertilization or fertilized and cultured *in vitro.* Both COCs and embryos were later permeablised and stained for evaluation. Illustration: Johan Hallberg

Media

All reagents were obtained from Sigma (Sigma Aldrich, Stockholm, Sweden) if not otherwise specified and media were prepared on regular basis.

After aspiration COCs were washed in search-medium consisting of hepes-buffered TCM 199 (M 7528) with supplement of 0.2 % w/v Albumin, Bovine 96-99%, BSA (Fraction V) (A3311) and 50 µg/ml Gentamicin-sulphate (G1264). Maturation-medium for *in vitro* maturation consisted of TCM 199 (M2154) with addition of 0.4 % w/v Albumin, Bovine 96-99%, BSA (Fraction V) (A3311), 0.68mM L-Glutamine (G8540), 50 µg/ml Gentamicin-sulphate (G1264), 0.5 µg/ml FSH and 0.1 µg/ml LH (Stimufol, Partnar Animal Health). To H and L treatments, bovine insulin (I5500) was added according to protocol.

For fertilization, 3 media were used; capacitation-medium for selection of motile sperm, wash-2 medium for handling of oocytes and fertilization-medium for *in vitro* fertilization.

Fertilization-medium consisted of 114 mM sodium chloride (S5886), 3.19 mM potassium chloride (P5405), 25.88 mM sodium bicarbonate (S5761), 0.29mM sodium phosphate (S5011), 0.49 mM magnesium chloride (M2393), 2 mM calcium chloride (C7902), 10 mM HEPES (H3375), 1 µl/ml phenol red (P0290), 0.5 µM sodium pyruvate (P4562), 0.03 µl sodium lactate (L7900), 50 µg/ml gentamicin-sulphate (G1264), 0.6% w/v Albumin, Bovine Serum, Fatty Acid Free (A7030) with addition of 3 µg/ml Heparin (H3149) and PHE giving a final concentration of 1.7 mM sodium chloride (S5886), 10 µM hypothaurine (H1384), 20 µM pencillamine (P4875), 1.5 µM epinephrine (E1635), 42 µM and sodium metabisulphite (S9000).

Capacitation medium consisted of 110 mM Sodium Chloride (S5886), 2.68 mM Potassium Chloride (P5405), 25 mM Sodium bicarbonate (S5761), 0.29 mM Sodium phosphate (S5011), 0.49 mM Magnesium chloride (M2393), 5 mM HEPES (H3375), 1 µM sodium pyruvate (P4562), 0.03 µl sodium lactate (L7900), 1.23 µg/ml glucose (G6152), 50 µg/ml gentamicin-sulphate (G1264) and 0.6 % w/v Albumin, Bovine 96-99%, BSA (Fraction V) (A3311).

Wash-2 media consisted of 114mM sodium chloride (S5886), 3.19 mM potassium chloride (P5405), 2 mM sodium bicarbonate (S5761), 0.29mM sodium phosphate (S5011), 0.49 mM magnesium chloride (M2393), 2 mM calcium chloride (C7902), 10 mM hepes (H3375), 1 µl/ml phenol red (P0290) with addition of 0.5 µM sodium pyruvate (P4562), 0.03 µM sodium lactate (L7900), 50µg/ml gentamicin-sulphate (G1264) and 0.3% w/v Albumin, Bovine 96-99 %, BSA (Fraction V) (A3311).

For *in vitro* culture of oocytes and embryos modified synthetic oviductal fluid (mSOF) was used. mSOF consisted of 0.11 M sodium chloride (S5886), 7 mM potassium chloride (P5405), 1.19 mM potassium phosphate monobasic (P5655), 25 mM sodium bicarbonate (S5761), 0.33 mM pyruvic acid sodium Salt (P4562), 1 mM L-glutamine (G8540), 0.171 mM calcium chloride (C7902), 1.5 mM glucose (G6152), 110 mM sodium lactate (L7900), 0.49 mM magnesium chloride (M2393) with addition of MEM non-essential amino-acids solution (100x) (M7145), amino acids solution (50x)(B6766), 0.4 w/v Albumin, bovine serum,

minimum 98 % (FAF: Fatty acid free) and 50 µg/ml gentamicin-sulphate (G1264). Embryos were cultured in 500 µl mSOF medium and covered with 300 µl paraffin-oil, Ovoil (Vitrolife AB, Gothenburg, Sweden) during culture.

Embryos/oocytes were fixed in 2 % PFA overnight and stored in PBS (147 mM sodium chloride (S5886), 2.7 mM potassium chloride (P5405), 1.5 mM potassium phosphate dibasic (P5655) and 1.4 mM sodium phosphate monobasic (VWR Laboratories, Stockholm, Sweden)) with addition of 1 % PVA (P8136) until staining.

In Vitro Production of Embryos

Bovine ovaries were collected immediately after slaughter from cows and heifers with unknown breed, age or reproductive status. Ovaries were transported 3-4h to the laboratory in 0.9% (w/v) NaCl solution at 29.0-32.5°C. COCs was aspirated from 3-8 mm or <3 mm follicles with an 18 g needle and a 5 ml syringe. COCs collected were rinsed through search-medium and quality was assessed through light microscope according to commonly used criteria, Grade 1: Compact cumulus investment with several layers CCs, the ooplasm is homogenous and the colour is light and transparent. Grade 2: As grade 1 but with coarse appearance, may not have as compact and as many layers of CCs as grade 1 and can have a darker zone in the periphery of the oocyte. Grade 3: Total COC appears darker then Grade 1 and 2, the cumulus complex is less compact and the ooplasm has irregularities or dark clusters. Grade 4: Expanded cumulus investment (Gordon, 2003, p 105)

COCs with supreme grades (Grade 1(-2) depending on the batch quality) were incubated for maturation and matured *in vitro* in maturation-medium randomly divided into three homogenous groups with 500 µl media, treated with high (H), low (L) or no (Z) insulin-treatment respectively. Oocytes were incubated at 38.5°C for 22 hours in 5 % CO_2, 5 % O_2 and 90 % N_2 with maximal humidity.

Expanded oocytes were prepared for fertilization by pipetting in wash-2-medium until 3-5 CC-layers remained. After washing the oocytes were fertilized *in vitro* or removed from culture for fixing and staining. Oocytes for fertilization were thereafter rinsed through fertilization-medium and fertilized *in vitro* with bovine sperm. Semen from a dairy bull of SRB breed with proven field and *in vitro* fertility was used (3-1716 Sörby).

Bovine semen (2 straws of 200 µl) was used. The spermatozoa were placed in the base of a tube with capacitation-medium and incubated in 5 % CO_2 incubator for 45 minutes. The top layer of capacitation-medium was suspended and centrifuged at (300 × g) for 5 min. Top layer was removed and sperm were after this diluted in fertilization-medium and counted. Sperm-solution was added to prepared oocytes for fertilization *in vitro*. Fertilization was done at a concentration of 1 x10^6 sperm per ml and incubated at 38.5°C for 22 hours in 5 % CO_2 + 5 % O_2 + 90 % N_2 with maximal humidity.

After fertilization *in vitro* zygotes and unfertilized oocytes were rinsed through mSOF-medium and all cumulus-cells remaining were removed. Wells with presumed zygotes were covered with Ovoil.

Presumed zygotes were cultured in mSOF media until day eight post fertilization. Cleavage rates and rate of cleaved above two cells was assessed at 44 hours post fertilization *in vitro.* Blastocysts were evaluated through stereomicroscope at day seven and day eight post fertilization. At day eight blastocysts were moved from culture for fixation and staining.

Staining

Matured oocytes and day 8 blastocysts *in vitro* were fixed and stained. Oocytes/embryos were fixed in 2 % PFA overnight in 4°C and then washed five times and kept in PBS with 1 % PVA in 4°C for 1-2 days before staining. Oocytes/embryos were then treated with 0.25 % Triton (X-100) during 20 min for permeabilization. After staining with TUNEL and nuclear stain, the oocytes and embryos were mounted on black well plates with Vectashield mounting medium (Vector Laboratories, Peterborough, United Kingdom) and sealed with nail varnish.

TUNEL staining

TUNEL assay was executed according to manufacturer protocol; all reagents except BSA, PBS were provided from the manufacturer. After each step, cells were washed in PBS if not otherwise mentioned.

Permeabilized cells were treated with terminal deoxynucleotidyl-transferase (TdT) during 10 min in RT, cells were incubated for 60 min in 37°C protected from light with TdT containing EdUTP Nucleotide mixture. After treatment, cells were washed in 3 % w/v Albumin, bovine serum, BSA (A4503) twice for three minutes. The Click-it reaction when the fluorocrome is attached to the apoptotic DNA-stains was after this executed, the Click-it reaction mixed with the Click-it additive and incubated for 30 min in RT protected from light.

Extensive DNA-fragmentation was induced in positive controls by incubation 30 min in RT with DNase I *(Figure 6)*. Negative control was emitted by excluding TdT from the reaction.

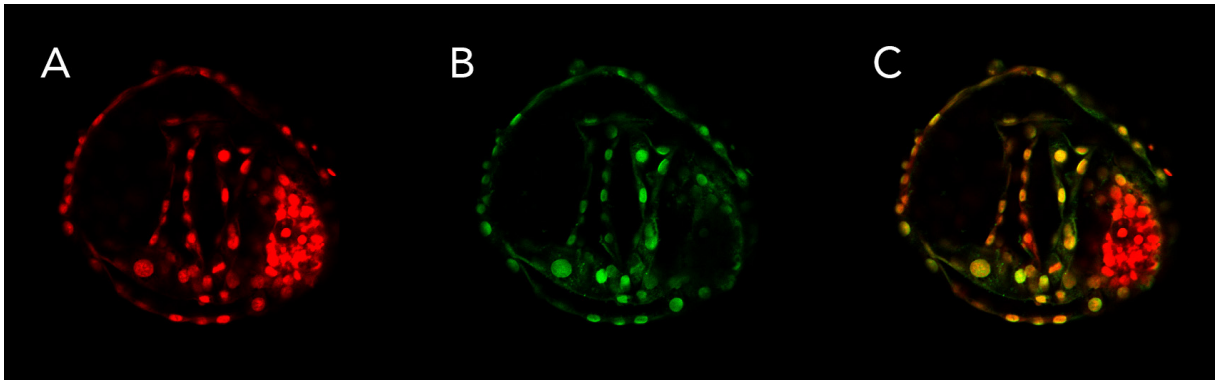

Figure 6. *Visualisation of apoptotic cells through the TUNEL-stain.* Positive control created through DNase treatment. Blastocyst is further stained with DraQ-5 nuclear stain and Click-it TUNEL apoptotic assay. Confocal picture showing nuclei marked red (A), apoptotic cells marked green (B) and red and green channel overlapping (C). Note the lack of apoptotic stain in the compact cell mass of the ICM. Confocal image, filter used to enhance picture quality. Image: Johan Hallberg & Ida Lindgren

On the positive controls, different permeabilisations with incubation with Triton X 100 in RT were tested. 0.25 % Triton X 100 for 20, 40 and 60 min and 0.5 % Triton X 100 for 20, 40 and 60 min.

Nuclear staining

After the TUNEL-reaction cells were stained with DraQ-5 (BioNordika, Stockholm, Sweden) nuclear stain for 20 min in RT protected from light in 2 µM DraQ-5 solution, followed by triple wash in PBS with 1 % PVA. One positive control was counter-stained with Hoechst 33342 nuclear stain for 20 min in RT protected from light.

Batch information

In all batches, the quality of the oocytes was assessed through previously mentioned standards and the average oocyte quality was assessed and graded subjectively from grade 1-2. In total, twelve batches were produced and eight of these were used in the study. Four batches were excluded due to insufficient COCs of Grade 1-2 resulting in too small groups and with insufficient blastocyst rate in one or two treatment groups at day eight.

The handling of the batches did not differ between the excluded and included batches, the temp at arrival to the lab, the aspiration time and the time from aspiration to maturation did not differ (*Table 1*). Though, in the excluded group, the subjective assessment of the oocyte quality was of lower grade than in the included groups. There were also less oocytes chosen for maturation per ovary in the excluded groups, and in total there were almost one third less oocytes set for maturation in the excluded batches (*Table 1).*

Table 1. *Batches*

Parameter, mean values	***Included***	***Excluded***
Number of ovaries	88	80.5
Temp at arrival (°C)	31.4	31.2
Aspiration time (min)	49.4	51.3
Aspiration to maturation (min)	79.3	78.8
Quality (manually assessed)	1.5	1.8
Total number of oocytes set for maturation	123.9	77.5
Total number of oocytes per ovary	1.4	1.0

Table 1: *Batches.* Mean values of parameters of included (n=8) and excluded (n=4) batches in the study. Aspiration time is time during which the oocytes was aspirated from the ovaries, Aspiration to maturation is the time where oocytes was found and selected, and divided into groups for maturation, Quality is the subjectively assessed average quality of the batch and total amount of oocytes set for maturation is the total amount of oocytes in the three groups of one batch

Evaluation

A total number of *n=132* matured oocytes divided in three groups from *n=8* batches were fixed and stained. A total number of *n=230* blastocysts from *n=8* batches were used for blastocyst development statistics, *n=201* blastocysts from *n=7* batches were used for fixation and staining, one batch excluded due to failure of positive control.

Assessing development

Data of cleaved oocytes was assessed 44h post fertilization by evaluation through light microscope. Cells dividing in two cells were considered cleaved, and cells divided into more than two cells were considered CA2.

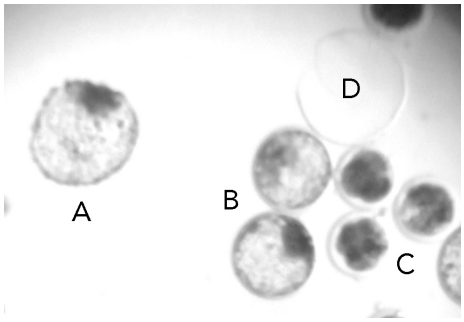

Figure 7. *Day eight blastocysts*. Stage and grade are assessed under light microscope. Picture showing expanding blastocysts (B), hatched blastocyst (A) with empty zona pellucida (D) and degenerated or not fully developed blastocysts (C). Image from lightmicroscope. Image: Johan Hallberg & Ida Lindgren

During the last days of IVP the stage and grade of the embryos produced *in vitro* are assessed. This was done through light microscope according to given standards which follows;

Stage; Embryos are graded according to development stage from 1-3. The most developed embryos are given the highest stage (3). Stage 1 is early blastocysts or 50 % blastocyst. These have an intact zona pellucida, and an antrum forming inside the embryo without having started to expand, hence the zona pellucida has not yet thinned out. Stage 2 embryos are expanding or expanded blasocysts who has started to expand which mainly can be seen on the zona pellucida thinning out. The antrum is bigger than in the Stage 1 blastocysts but also depending on grade (see below). Stage 3 embryos are hatching or hatched meaning the zona pellucida has cracked open and parts of - or the whole embryo has pressed through.

Grade; Embryos of all stages were graded from 1-4, with 1 being the most excellent embryo and 4 being a degraded or dead similar. Grade 1; Embryo with symmetrical and spherical mass. Individual cells are uniform in size, shape and density. If there are irregularities, they are relatively small. The zona pellucida (if present) should be visible smooth and have no irregularities. Grade 2: Fair. The embryo has moderate irregularities in shape and/or size, density and color. The compact cell cluster representing the inner cell mass includes about 50 % of total blastomeres. Grade 3: Poor. The embryo has irregularities in shape, size, color and/or density of the whole embryo of individual cells that are major. ¼ of the embryonic cell mass is visible as an intact viable cell-cluster. Grade 4: Dead or degenerating, here is also nonviable one-cell embryos and oocytes include (International Embryo Transfer Society, 2010, p.89).

Assessing results from stainings

Oocytes and embryos were mounted and scanned through Leiss LSM 510 confocal microscope for analysis.

The definition of a nucleus was structures stained by the DraQ-5 component, interpreted as red by the confocal microscope. Apoptotic cells was cells both stained by DraQ-5 component and TUNEL-stain showing or not showing apoptotic appearance as this has been shown to have little correlation between true apoptotic cells (Gjørret et al. 2003). TUNEL-positive nuclei not stained DraQ-5 were not included as apoptotic cells. Small fragments in the same area stained both with Draq-5 and TUNEL-stain was interpreted as fragments from one singular nuclei (*Figure 8*)

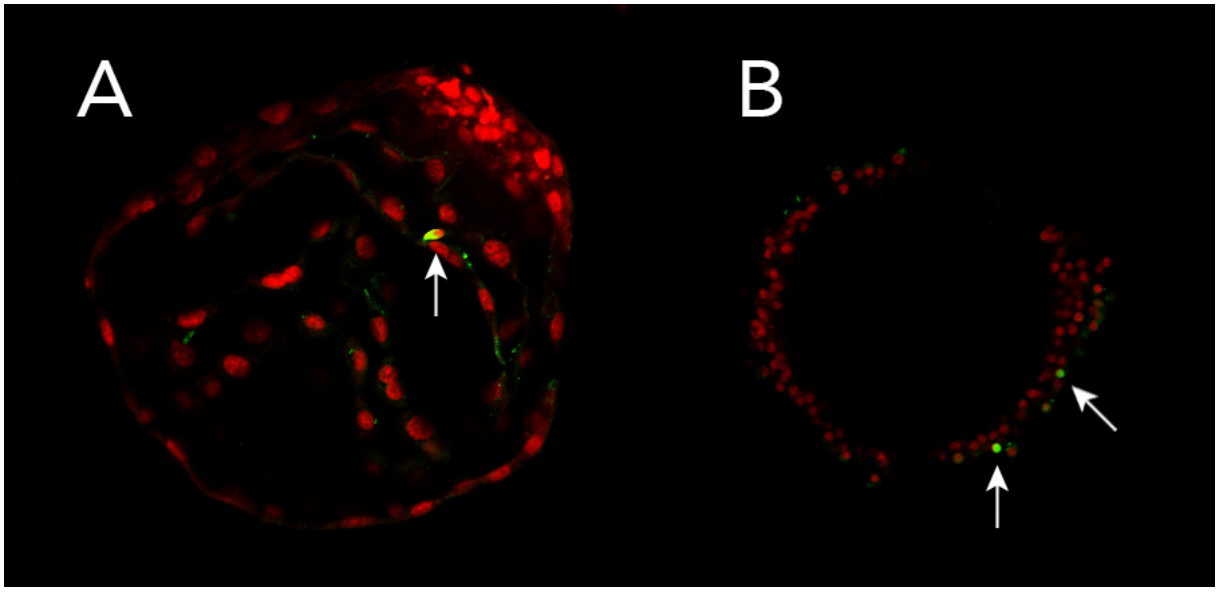

Figure 8. *Apoptotic cells in blastocysts and COCs*. Blastocyst (A) and COC (B) stained with DraQ-5 nuclear stain and Click-it TUNEL assay showing apoptotic blastomeres and cumulus cells (arrows). Confocal image, filter used to enhance picture quality. Image: Johan Hallberg & Ida Lindgren

Blastocysts were scanned in eight levels, three levels separated by one level was used for analysis of the blastocysts. The scanned level separating the used scans prevented cells to occur in two images. Nuclei and apoptotic nuclei in the three images used, were counted manually by the same person in all blastocysts. The nuclei counted (apoptotic and total) were all blastomeres visible in mentioned images, independent of origin in ICM or Tb.

After counting nuclei and apoptotic nuclei, the blastocysts were assessed concerning ICM. In blastocysts with ICM visible as an even compact cluster of cells, the grade was 1, and in blastocysts were no ICM could be identified or the cell cluster interpreted as ICM were spread out over 50 % of the embryo were given grade 0.

COCs were scanned in five levels, two levels separated by one level was used for analyses of the COC. The scans separating the used images prevented cells to occur in two images. Nuclei

and apoptotic nuclei were counted manually by the same person in the outer two layers of the COCs. All nuclei were not counted, as the TUNEL-stain did not reach more then 3-5 cell-layers. During analysis of the scans, the cumulus-complex grade (CC-grade) was assessed, Grade 1 for oocytes with <3 layers of cumulus cells, Grade 2 for 2-5 layers of cumulus cells and Grade 3 for >5 layers of cumulus cells.

During confocal microscopy of the oocytes, the presence of a polar body was investigated. Oocytes were defined as in M2 stage or not in M2 stage depending on appearance of a polar body, results were classified as missing if M2-stage could not be identified through confocal microscopy (*Figure 9)*.

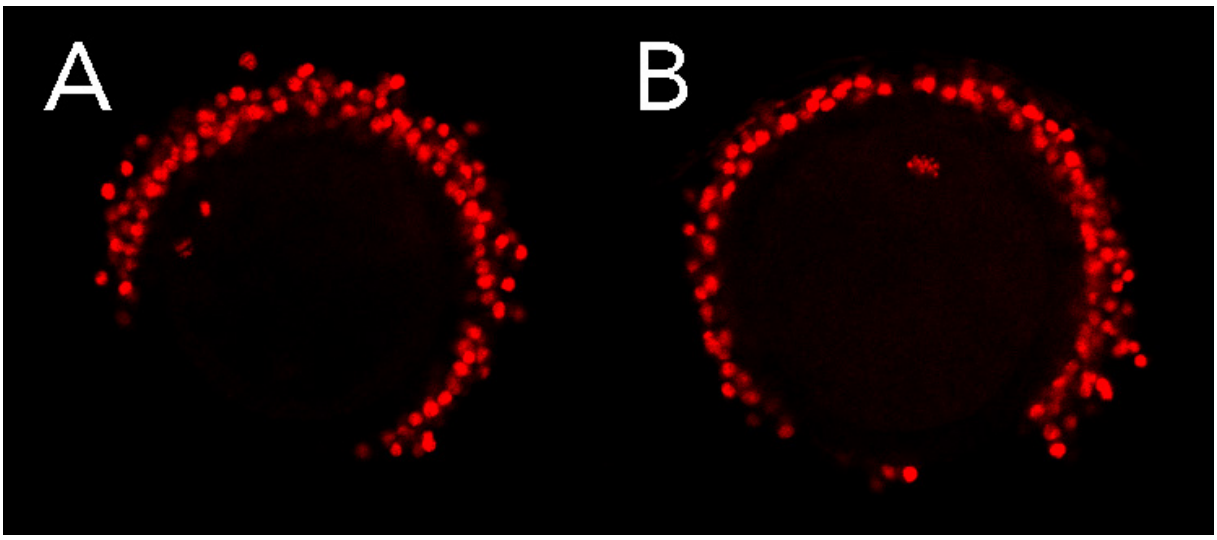

Figure 9. *Detection of meiotic stage.* COCs were examined for presence of polar body indicating M2 stage of the oocyte. COCs stained with DraQ-5 nuclear stain, A with polar body present and B with polar bodies absent. Confocal image, filter used to enhance picture quality. Image: Johan Hallberg & Ida Lindgren

Photobleaching of staining

Sample was taken from batches stained seven, five and three weeks before to test photobleaching of stainings. The images were taken with the same settings as for the image-scan taken at the time of the first analysis of the oocytes or embryos. There was no difference on the images scanned after seven, five and three weeks regarding bleach of the stainings visible to the human eye (*Figure 10*).

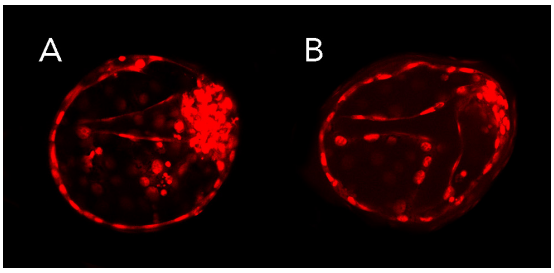

Figure 10. *Photo bleaching of staining.* Picture showing blastocyst stained with DraQ-5 nuclear staining photopraphed through confocal microscope. Same settings are used on both picture but with slightly different focus. A is taken after staining, B is photopraphed after seven weeks of storage in 4°C partly protected from light. Confocal image, filter used to enhance picture quality. Image: Johan Hallberg & Ida Lindgren

Statistical analysis

Analysis of embryonic development was made by studying the following parameters; cleaved from total fertilized and transferred, cleaved above 2 cells from total fertilized and transferred, blastocyst-rate from total fertilized and transferred and from cleaved on day 7 and 8, blastocyst stage and grade. The analysis of the characteristics of the embryos was made on the following parameters; Amount of nuclei, percentage of apoptotic cells, Compact ICM (1/0) visible through confocal microscope. The analysis of oocyte characteristics was made on the following variables; Percentage of apoptotic cells, CC-grade and presence of polar body (1/0).

All percentages were arc sinus squareroot transformed before making statistical analyzes. Transformed values on effect of treatment on variables above were analysed by ANOVA (proc GLM SAS v.9.2). Post ANOVA comparisons between H+L group vs Z were performed by using the contrast option under GLM (Scheffe Test).

Same procedures were used to test the effect of treatment and visualization of compact ICM (1/0) on the percentages of apoptotic cells. The total number of nuclei was tested through the same model (without any transformation).

Same procedures were used to test the effect of treatments and CC-grade on percentages of apoptotic cells in the cumulus complex. Log linear models (proc logistics SAS ver 9.2) were used to test the effect on treatments on the CC-grade. LSmean with SE are presented. P-values <0.05 were considered significant.

Mean values were calculated of the presence of a polar body (oocytes reaching M2-stage).

RESULTS

In this experiment 132 matured COCs from eight batches were analyzed for presence of apoptosis and polar body (1/0). 230 blastocysts from eight batches were assessed on day seven and eight regarding stage and grade. 201 blastocysts from day 8 *in vitro* from seven batches were stained and analyzed for presence of apoptosis. One batch of blastocysts was excluded from the TUNEL-staining due to failure of positive control.

There was no difference between the individual batches nor within the same treatments between different individual batches in any of the parameters investigated (p>0.05).

Embryo development

The cleavage rate calculated from fertilized and transferred oocytes was 85-89% and the cleavage rate above two cells from transferred oocytes was 73-75% at 44h post fertilization with no difference between the groups (p>0.05) *(Figure 11)*.

Insulin tended to impair the blastocyst development on day 7 and day 8. Differences between groups were more significant when comparing blastocyst development with cleaved oocytes rather than all fertilized and transferred oocytes (Z: 0.85±0.02, L: 0.85±0.02, H:0.89±0.02, p>0.05). The difference was significant when comparing blastocyst rate on day 7 from cleaved oocytes between all the groups (p=0.05) *(Table 2)*.

When testing the contrasts (both insulin treated groups together versus the control), significant differences in blastocyst development rates by day 7 were found from development rates by day 7 calculated from cleaved and all fertilized and transferred oocytes *(Table 2)*

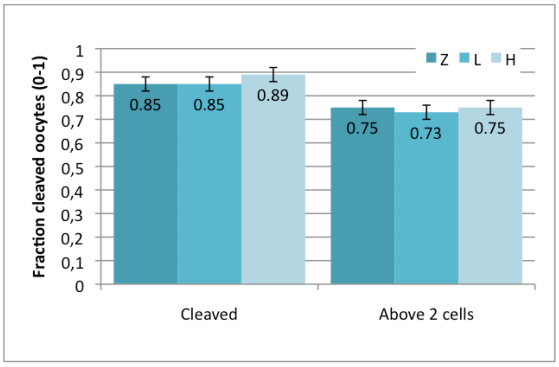

Figure 11: *Fraction cleaved oocytes at 44h post fertilization.* Fraction cleaved oocytes after maturation *in vitro* under different insulin conditions (Z= 0 µg/ml L= 0,1 µg/ml H 10 µg/ml). The difference between groups was not significant (p>0.05)

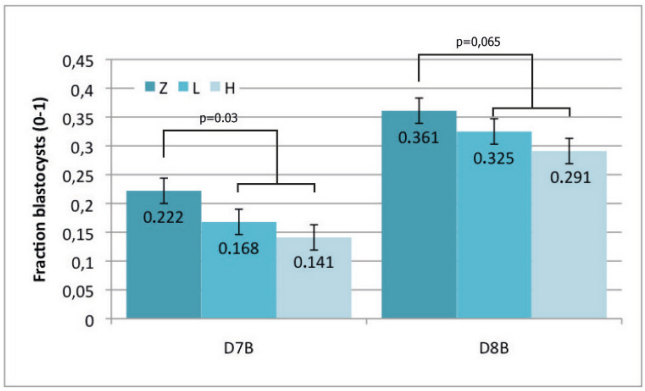

Figure 12: *Fraction blastocysts.* Fraction blastocysts after maturation *in vitro* under different insulin conditions (Z= 0 µg/ml L=0.1 µg/ml H=10 µg/ml). Figure showing blastosysts assessed at day seven (D7B p=0.03) and day eight (D8B p=0.065).

Table 2. *Development statistics*

	Z	L	H	Sign[a]
Cleaved from fertilized	0.85 (±0,02)	0.85 (±0.02)	0.89 (±0.02)	ns
CA2 from fertilized	0.75 (±0,03)	0.73 (±0,03)	0.75 (±0,03)	ns
D7B 7 from fertilized	0.19 (±0,02)	0.14 (±0,02)	0.13 (±0,02)	p=0.08
D8B from fertilized and transferred	0.30 (±0,02)	0.28 (±0,02)	0.26 (±0,02)	p=0.2
D7B from cleaved	0.22 (±0,02)	0.17 (±0,02)	0.14 (±0,02)	p=0.05
D8B from cleaved	0.36 (±0,02)	0.33 (±0,02)	0.29 (±0,02)	p=0.1
D7B from fertilized (Z vs H+L)[b]				p=0.036
D8B from fertilized (Z vs H+L)[b]				p=0.1
D7B from cleaved, (Z vs H+L)[b]				p=0.026
D8B from cleaved, (Z vs H+L)[b]				p=0.065

Table 2. *Development statistics.* Embryo development assessed 44h post fertilization (cleaved and cleaved above 2 cells) and on day 7 and 8 of in vitro culture. Presented are variables and percentages from the experiment, results are presented as LSmean (±SE). Values are considered significant with $p<0.05$.

[a]Corrected values, arc sin of square-root

[b] Control group compared to both insulin treated groups

Blastocysts

There was a tendency that insulin treated groups compared to the control group had developed into earlier development stages and the blastocyst has developed into inferior grades, however, the difference was not significant ($p>0.05$). When testing the contrast (treated groups compared to control group) the difference was significant for stage ($p=0.02$) and grade ($p=0.03$) (*Table 2)*

Blastocysts nuclei

The mean number of nuclei in the blastocyst (ICM+Tb) was not significant between the the treated groups and the control group with this staining (Z:107.5, L:96.2, H:106.5) ($p=0.22$). Proportion of blastocysts with compact visible ICM was not different between the groups ($p>0.05$).

There was a tendency that insulin decreased the amount of apoptotic nuclei when testing the contrast on treated groups together on the control-group, but the difference was not significant ($p=0.08$) and the standard error was relatively high (Z: 0.021±0.003, L: 0.026±0.003, H: 0.028±0.003).

Comparing the blastocyst with visible compact ICM independent of treatment to those without visible compact ICM, blastocysts with compact ICM had more nuclei in total (123±1.7 vs 84±3.8, $p<0.01$). Blastocyst with compact ICM had less apoptotic cells then

blastocyst where no compact ICM could be identified (0.016±0.003 vs 0.033±0.003, p<0.01) (*Figure 13 and 14*)

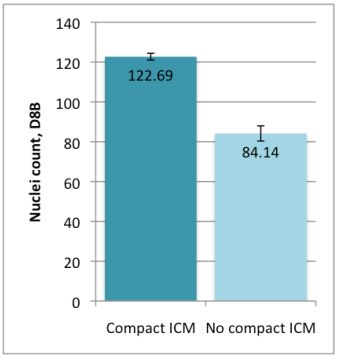

Figure 13: *Nuclei count.* Amount of nuclei in blastocysts at day eight compared between those with visible compact ICM to those without compact ICM. p<0.01

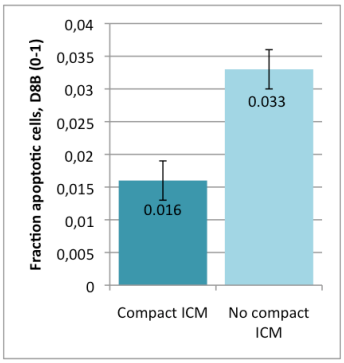

Figure 14: *Fraction apoptotics cells.* AI in blastocysts at day eight compared between those with visible compact ICM to those without. p<0.01

COC nuclei

The apoptotic index (AI) of cells in the outer two layers of the cumulus-complex of the oocyte was 5.2-7.7% (Z: 5.2±2.5% L: 3.9±1.6% H: 7.7±4.4%), the difference was not significant (p=ns). There was no correlation between the treated groups and control group comparing cumulus-complexes (*Figure 15*).

Oocytes assessed as in M2-stage was 31/33 in control group, 26/30 in L and 28/31 in H.

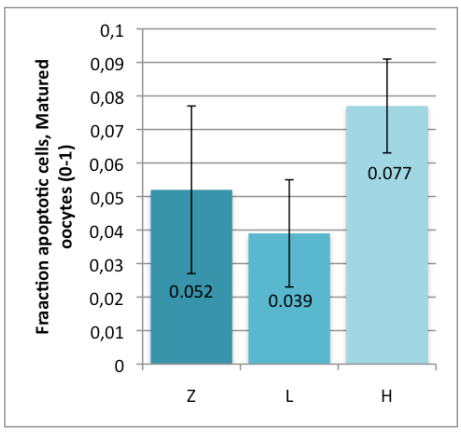

Figure 15: *Fraction apoptotic cells in COCs.* AI in the outer two layers of the cumulus complex. The difference between the groups was not significant (p>0.05).

Permeabilization

Different permeabilization technique did not increase the penetrance of the apoptotic stain in the positive control, however the staining did seem to stain more distinct when premeabilized during longer time/higher concentration.

DISCUSSION

Experimental design/IVP model

The aim of this study was to test the effect of insulin during maturation *in vitro* on abattoir-derived bovine COCs and further to evaluate a nuclear stain and an apoptotic detection kit using the TUNEL-method for COCs and embryos.

The study showed an impaired blastocyst development in insulin-treated groups compared to the control-group with no insulin. Further, there was a tendency that stage and grade of the embryos also were impaired in insulin-treated groups. The TUNEL-stain used did not penetrate the compact cell cluster of the COCs nor blastocysts sufficiently. The nuclear stain tested had adequate properties for use in COCs and embryos.

The IVP model has some advantages compared to *in vivo*-studies, making it a suitable model for early embryo development. It enables large numbers of oocytes and embryos generated for research and investigations under fixed conditions with minimal animal use. Further, the bovine model of IVP has shown similarities to early embryo development in humans, making the bovine model usable for basic research regarding early mammalian development (Gordon 2003, p.17).

Still, one should be aware that the IVP model does not fully correspond to the *in vivo* situation as the laboratory environment is a simplification of the environment in the reproductive tract influenced by the complexity of the mother organism. With this, there are both advantages and disadvantages. Advantages, as the environment *in vitro* is not influenced by as many factors as the corresponding model *in vivo* are making the experiment more controlled and focused on a singular thesis. Disadvantages as the results from *in vitro* models cannot be read into real life situations without particular interpretation, as for one, the maturation period of the oocyte differs significantly between *in vivo* and *in vitro* situations. In spite of this, the IVP model is a very good alternative when investigating a single factor during a specific period of embryo development. It is also useful as a pre-investigation technique before proceeding to *in vivo* studies.

Insulin

This insulin IVP model is a model studying the effect of insulin during maturation on bovine early embryo development until day eight, seeking eventual effect of insulin on embryo development and eventual toxicity in higher insulin concentrations used. In this study the oocytes was exposed to elevated insulin concentrations compared to levels measured in serum or follicular fluid *in vivo*. The level of insulin in the L group was comparable to physiological levels though still elevated and the insulin concentration in the H group was much elevated compared to serum/follicular fluid. The control group did not correspond to the *in vivo* dairy cow as the level of insulin in this group was zero. The aim of this model was to test the effect on treated groups versus the non-treated group (insulin versus no insulin) during maturation *in vitro*. The motivation behind increasing the levels of insulin beyond the physiological level was to compensate for the fact that the maturation *in vitro* occurs in higher speed compared to

the maturation *in vivo*. We wanted to investigate if there was a dose-effect or a switch in the influence pattern (positive or negative depending on the amount of insulin).

In this study, the effect on insulin had no significant effect on oocyte cleavage which does not contradict studies earlier showing elevated cleavage in insulin-treated groups compared to control group (Bowles & Lishman 1998; Gong *et al.* 2002). It is possible that an eventual positive effect of insulin on cleavage is masked due to general high cleavage-rates in all groups.

The blastocyst rate was impaired in insulin treated groups versus control group with significant values on D7B from transferred oocytes and from cleaved oocytes. This is consistent to other studies showing decreased blastocyst rates after insulin treatment during *in vitro* maturation or culture with concentrations at 10 µg/ml (Bowles & Lishman 1998; Laskowski *et al.* 2013). However, as mentioned before, contradictory studies have also shown an increased early embryo development to the morula stage by adding insulin to the culture medium (Matsui *et al.* 1995b).

In this study, no difference in number of nuclei could be seen between groups treated with insulin and not treated during maturation. This do not support previous studies showing an increased number of nuclei in blastocyst treated with insulin in maturation medium or culture medium (Bowles & Lishman 1998; Laskowski *et al.* 2013). The reason for the suggestion that insulin decrease the blastocyst rate while increasing the blastomere quantity in formed blastocysts may be explained by the action mechanisms of insulin. Increased differences between the groups treated and not treated with insulin have been shown when adding metabolites such as amino-acids and glucose to the medium, suggesting that the effect of insulin on embryos are mediated through elevated metabolism, hence the need for metabolites (Matsui *et al.* 1995a; Bowles & Lishman 1998).

The amount apoptotic cells in both COCs and blastocysts tended to be higher in the treated group compared to the control group, the results however were not significant. Increased apoptotic cells in the insulin-treated groups suggest that the blastocyst treated with insulin have more activity, consistent to suggestions that insulin may increase the number of nuclei of the blastocysts and hence the mitotic activity (Bowles & Lishman 1998; Laskowski *et al.* 2013).

Stainings

The staining used to detect apoptosis was the Click-it TUNEL apoptosis assay kit, used before on astrocytes and human retinal Müller cells (Wu *et al.* 2012; Wang e*t al.* 2012) but as far as known by author, not previously used and published on oocytes and/or embryos.

The kit did stain presumed apoptotic cells detectable with fluorescence microscopy in exciting/emission at 590/615 nm. Nuclei were interpreted as apoptotic when both nuclear stain (DraQ-5) and TUNEL assay stained the nucleus. Most TUNEL-positive nuclei also had apoptotic morphology, defined as pycnotic, more intense nuclear stain, compact chromatin in a decreased volume or fragmented nucleus (Gjørret *et al.* 2003; Rodríguez *et al.* 2006).

It was clearly visible that whole, or parts, of the presumed ICM was not stained with the TUNEL-assay, neither in the positive control nor in the other blastocysts stained in the study. This did not differ between different permeabilization techniques tested, independent of both concentration and duration. The ICM consists of a compact cell-cluster this could be the reason for the failure of the stain to penetrate the cells, as can the cells included in the ICM express some mechanism of evading the staining. However, as the same pattern with inability to stain compact cell-clusters was found in the COCs, it is reasonable to presume that the stain does not possess qualities to penetrate cell masses of this magnitude. As the mean amount of apoptotic cells in the blastocysts (2.1-2.8%) do conform with the apoptotic cells found in the trophoblast cells in blastocysts brought to day 8 of *in vitro* culture (1.8-2.4%) (Gjørret *et al.* 2003) there is a further suggestions that the stain did indeed stain the trophoblasts but did not reach the ICM. As the mean values of apoptotic cells were calculated from all nuclei in the blastocysts, not solemnly the trophoblasts however, the true amount of apoptotic cells in the trophoblasts was most likely higher.

In the COCs stained the same pattern could be seen. The TUNEL-stain did not seem to reach more then 3-5 cell-layers through the cumulus-complex making the evaluation of the nucleus of the oocyte impossible on apoptotic basis. The apoptotic index of the CCs was in this study between 4-8% in the outer most two layers, with no significant difference between the treated and non-treated groups (Z: 5.2% ± 2.5, L: 3.9% ± 1.6, H: 7.7% ± 1.4%). This consists with Warcych et al who showed in a study 2012 that most oocytes had less than 15 % apoptotic cells. The same study, also suggested that the AI did not correspond well to the *in vitro* maturation nor meiotic competence, making AI a questionable marker for oocyte competence (Warzych *et al.* 2013).

The nuclear stain used in this study had an optimal exciting and emission state used for the confocal microscope used. The staining have been used and evaluated on different cell-types, included bovine embryos/oocytes (Smith *et al.* 2000; Martin *et al.* 2007), though never tested in combination with TUNEL-stain instead of Hoechst 33342 for evaluating embryos/COCs. A frequently used nuclear staining for embryos and oocytes is the Hoechst nuclear staining (Roth & Hansen 2004; Adamiak *et al.* 2005; Rodríguez *et al.* 2006; Abraham *et al.* 2012). The DraQ-5 and Hoechst showed identical staining, suggesting that DraQ-5 as an adequate nuclear staining for embryos and oocytes. This is consistent with the pattern seen in other cell-types where DraQ-5 and Hoechst have been compared (Martin *et al.* 2005; Martin *et al.* 2007).

Stained blastocysts were stored in a dark box in 4°C, with exception when out for confocal microscopy. Blastocysts scanned three, five and seven weeks after staining with the same settings showed no signs on photobleaching regarding both DraQ-5 and the TUNEL-assay.

In vivo applications

Many studies implicate the complexity in the effect of insulin on reproduction, as the effect of insulin on early embryo development differs from the effect on maturation of the oocyte. It has been concluded how events occurring before ovulation not only determine the fate of the oocyte but also affect the outcome of fraction blastocyst developing *in vitro* (Lonergan *et al.*

2001). The parameters of profound importance of blastocyst quality *in vitro* however, are suggested to be events occurring after the zygote-stage (Lonergan *et al.* 2001; Gordon 2003, p.19).

In vivo models show that cows on insulinogenic (starch-rich) diets resume ovarian cyclic activity faster post partum (Garnsworthy et al. 2009) and the assumption is that elevated insulin-levels during oocyte-maturation may be of advantage at this period. The beneficial effect of elevated insulin levels through insulinogenic diets post partum might rather be an indirect effect due to the shortened period of NEB than a direct positive effect on oocyte maturation but this has to be further investigated.

Increased levels of insulin during oocyte maturation, however, has *in vivo* showed to reduce the oocyte quality (Adamiak *et al.* 2005), an assumption that is consistent with the results in this study.

It has also been shown in human studies that women with elevated serum-insulin caused by insulin-resistance have reduced fertility. Compared to healthy individuals the implantation rate was decreased while no difference could be seen in the IVP results before (IVM/IVF/IVC) (Chang *et al.* 2013).

CONCLUSION

This study showed a decreased blastocyst rate from oocytes treated with insulin during maturation *in vitro* suggesting that elevated insulin levels have a negative effect on early embryo development. This study could not show differences in apoptotic cells, neither in COCs nor in blastocysts.

The TUNEL-kit evaluated did not work satisfactory for staining of embryos and COCs with multiple cell-layers. The nuclear staining tested however, seem to be a good candidate for staining of blastomeres and COCs with a limited amount of cell-layers. The stain is easy to use and further does not photobleach after several weeks of storage.

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