UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

EMBRYO-MATERNAL INTERACTIONS LEADING TO EMBRYONIC DEVELOPMENT AND SURVIVAL IN THE BOVINE – ROLE OF PROGESTERONE AND PROSTAGLANDINS

Ana Catarina Belejo Mora Torres

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TESE DE DOUTORAMENTO EM CIÊNCIAS VETERINÁRIAS

ESPECIALIDADE DE CLÍNICA

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Chegada ao último ciclo de estudos, o doutoramento, a aprendizagem continuou intensamente… Aprendi novas metodologias, novas teorias, formas de delineamento experimental, uma abordagem sistemática aos métodos e à análise dos resultados obtidos e formas mais eficazes de comunicação com a comunidade científica. Mas para além de crescer na Ciência, ou também para ser uma cientista melhor, o doutoramento permitiu-me ainda aprender a ser mais paciente, a lidar melhor com as frustrações de semanas de trabalho por vezes infrutíferas, a trabalhar melhor em equipa e a entender diferentes pontos de vista, a procurar constantemente novas soluções para os problemas, a gerir melhor o meu tempo.

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Título da dissertação: Interações embrio-maternas relevantes para o desenvolvimento e sobrevivência embrionários em bovinos – papel da progesterona e das prostaglandinas

Resumo: Os objetivos desta tese foram: avaliar interações embrio-maternas esteroidogénicas e prostanoides no desenvolvimento e sobrevivência embrionárias; testar estratégias terapêuticas na transferência embrionária (TE) com vista ao aumento da sobrevivência embrionária.

Experiências *in vitro* (três capítulos experimentais) – embriões bovinos (Dia 7): i) revelaram transcrição de genes codificantes das enzimas das vias sintéticas da progesterona (P_4) e PGs (PTGS2, PGFS, PGES, StAR, P450scc,3β-HSD); ii) produziram estes mediadores (P_4, $PGF_{2\alpha}$, PGE_2) para o meio de cultura; iii) apresentaram um aumento significativo dos níveis de transcrição destes genes (à exceção da StAR) associado à primeira diferenciação celular embrionária; iv) derivados de dadoras de oócitos pré-púberes revelaram níveis de transcrição dos genes mencionados similares aos de embriões de dadoras cíclicas (à exceção dos níveis de transcrição para a 3β-HSD, tendencialmente mais elevados em embriões provenientes de fêmeas cíclicas). Adicionalmente, v) num modelo de co-cultura de células lúteas desenvolvido, estas exerceram um efeito embriotrófico, aumentando significativamente a taxa de desenvolvimento e qualidade embrionárias; porém, este efeito não foi associado a aumento na transcrição génica ou produção de P_4, $PGF_{2\alpha}$, PGE_2; vi) Embriões em co-cultura com células lúteas não exerceram um efeito luteotrófico nas células; e vii) o uso de óleo mineral na cobertura dos poços de cultura exerceu um efeito embriotrófico, mas absorveu P_4 do meio.

Experiências *in vivo* (dois capítulos experimentais) – novos modelos *in vivo* - embriões de baixa competência de desenvolvimento (hemi-embriões) e recetoras sub-férteis (vacas leiteiras de alta produção) ou com fertilidade alta (novilhas leiteiras virgens) - foram usados na avaliação do efeito na sobrevivência embrionária e nas concentrações plasmáticas de P_4 e PSPB das recetoras, de tratamentos, na TE, com hCG ou carprofen. Concluiu-se que: i) o tratamento com hCG induziu a formação de CLs secundários, aumentou as concentrações plasmáticas de P_4, a taxa de sobrevivência dos hemi-embriões e as taxas de gestação das recetoras (em vacas). Os embriões foram resgatados para além do reconhecimento materno da gestação (RMG), mas a sobrevivência embrionária posterior, o crescimento até à implantação e a secreção placentária de PSPB até ao Dia 63 de gestação (testados em vacas) não foram afetados; ii) a sobrevivência embrionária após o RMG não está diretamente dependente das concentrações de P_4 maternas; iii) o tratamento com o carprofeno não afetou significativamente as concentrações de P_4 ou a sobrevivência embrionária, mas diminuiu o efeito luteotrófico da hCG.

Palavras-chave: embrião; células lúteas; progesterona; prostaglandinas; bovino.

Thesis title: Embryo-maternal interactions leading to embryonic development and survival in the bovine – role of progesterone and prostaglandins

Abstract:

The objectives of this thesis were to evaluate steroidogenic and prostanoid embryo-maternal interactions leading to embryonic development and survival in cattle, and to evaluate therapeutic strategies at embryo transfer (ET) designed to enhance embryo survival.

In vitro experiments (three experimental chapters) - bovine early (Day 7) embryos i) had transcription of genes coding for enzymes progesterone (P_4) and of prostaglandins (PGs) synthesis pathways (StAR, $P450_{scc}$,3β-HSD, PTGS2, PGFS, PGES); ii) produced these mediators (P_4, $PGF_{2\alpha}$, PGE_2) into culture medium; iii) had a significant increase in transcription levels of the above genes (except StAR) associated to first embryonic cellular differentiation; iv) derived from pre-pubertal oocyte donors had transcription levels of the above genes similar to those of embryos derived from post-pubertal cyclic heifers (except for 3β-HSD, which tended to be higher in embryos from cyclic heifers). Additionally, v) in a developed luteal cells (LC) co-culture model, LC induced an embryotrophic effect, significantly increasing blastocyst yield and quality; however, this embryotrophic effect was not associated with an increase in embryonic gene transcription or production of P_4, $PGF_{2\alpha}$, PGE_2; vi) Embryos co-cultured with LC did not exert a luteotrophic effect upon the cells; and vii) oil overlaying of culture wells exerted an embryotrophic effect, but absorbed P_4 from culture medium.

In vivo experiments (two experimental chapters) - novel *in-vivo* models considering poor developmental competence embryos (demi-embryos) and either sub-normal fertility recipients (lactating high-yielding dairy cow) or high fertility recipients (virgin dairy heifers) were used to evaluate the effect of hCG and carprofen treatment at embryo transfer on embryo survival and on plasma P_4 and PSPB concentrations of recipients. Conclusions were that: i) treatment with hCG induced formation of secondary CL, increased plasma P_4 concentrations, survival rate of demi-embryos and pregnancy rate of recipients (only in cows). Embryos were rescued beyond maternal recognition of pregnancy (MPR), but later embryonic survival, growth until implantation and placental PSPB secretion until Day 63 of pregnancy (only tested in cows) were not affected; ii) embryonic survival following MRP is not under direct dependency of maternal P_4 concentrations; iii) treatment with carprofen had no significant effect on plasma P_4 concentrations and embryonic survival, but decreased the luteotrophic effect of hCG.

Keywords: embryo; luteal cells; progesterone; prostaglandins; bovine

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3β-HSD – 3 Beta hydroxysteroid dehydrogenase

AI – artificial insemination

ANOVA – analysis of variance

B2M – beta2-microglobulin

BL – blastocyst

BOEC – bovine oviduct epithelial cells

BRL cells – Buffalo rat liver cells

BSA – bovine serum albumin

cDNA – complementary DNA

CL – corpus luteum

CM – compact morula

COC – cumulus-oocyte complex

COX-2 – cyclooxygenase-2

CRL – crown-rump length

DMEM – Dulbecco's modified Eagle's medium

DNA - deoxyribonucleic acid

E_2 – estradiol- 17β

early CL – early luteal phase CL

EBL – expanded blastocyst

EFM – early fetal mortality

EGA – embryonic genomic control

EGF – epidermal growth factor

ELISA – enzyme-linked immunosorbent assay

EMM – early embryonic mortality

EP – Prostaglandin E_2 receptor

ER – estrogen receptor

ET – embryo transfer

FGF – fibroblast growth factor

FP – prostaglandin $F_{2\alpha}$ receptor

FSH – Follicle-stimulating hormone

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GE – uterine glandular epithelia

GM-CSF – granulocyte-macrophage-colony stimulating factor

GnRH – Gonadotrophin-releasing hormone

HBSS – Hank's buffered salt solution

hCG – human chorionic gonadotrophin

HDL - high density lipoprotein

ICM – inner cell mass

IETS – International Embryo Transfer Society

IFN-τ - interferon tau

IGFBP – insulin-like growth factor binding proteins

IGF-I – insulin-like growth factor I

IGF-II - insulin-like growth factor II

IVP – *in vitro* production

LC –luteal cells

LDL – low density lipoprotein

LE – uterine luminal epithelium

LEM – late embryonic mortality

LH – luteinizing hormone

LIF – leukemia inhibitory factor

LLC – large luteal cells

LPS – lipopolysaccharide

M – Molar

MET – Maternal-to-embryonic transition

MHC – major histocompatibility complex

mid CL – mid luteal phase CL

mPR – membrane bound P_4 receptor

mRNA – messenger ribonucleic acid

MRP – maternal recognition of pregnancy

NSAIDs – non-steroidal anti-inflammatory drugs

O_2 – oxygen

OA – ovarian artery

OXT - oxytocin

OXTR – oxytocin receptor

P_4 – progesterone

$P450_{scc}$ – cytochrome 450 side-chain cleavage

PAG – pregnancy-associated glycoproteins

PBR – Peripheral-type benzodiazepine receptors

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PDGF – platelet-derived growth factor

PG – prostaglandin

PGE_2 – prostaglandin E_2

PGES – prostaglandin E_2 synthase

$PGF_{2\alpha}$ – prostaglandin F_2 alpha

PGFS – prostaglandin F_2 alpha synthase

PGH_2 – prostaglandin H_2

PGI_2 – prostaciclin

PR – progesterone receptor

PKA – protein kinase A

PKC – protein kinase C

PSPB – Pregnancy-specific protein B

PTGS1 – Prostaglandin-endoperoxide synthase 1

PTGS2 – Prostaglandin-endoperoxide synthase 2

RIA – radioimmunoassay

RT – reverse transcriptase

SEM – standard error mean

sGE – superficial glandular epithelia

SLC – small luteal cells

SO – superovulation

SOF – synthetic oviductal fluid medium

ß actin – beta actin

StAR – steroidogenic acute regulating protein

TCM-199 – Tissue Culture Medium 199

TE –trophectoderm cells

TGF – transforming growth factors

TNFα - tumor necrosis factor alpha

UOV – utero-ovarian vein

v/v – volume fraction

w/v – mass concentration

YBL – young blastocyst

ZP – zona pellucida

INTRODUCTION – theme presentation, justification and objectives

Cattle are an essential worldwide source of animal protein. In Portugal, cattle dairy and meat products represent more than half of the total national animal production (28,1% and 25,0%, respectively; Ministério da Agricultura, do Mar, do Ambiente e do Ordenamento do Território, 2007). In the last decades, dairy cows have been selected to increase their production traits, and this has been associated to a decline in fertility (Rodriguez-Martinez *et al.*, 2008). In Portugal, a steady decline in fertility of dairy cows was also reported (Rocha *et al.*, 2001; Rocha *et al.*, 2010).

Embryonic mortality is the most prevalent component associated to the fertility decrease in the modern high-yielding dairy cow (Leroy *et al.*, 2008). Most embryo losses occur between fertilization and maternal recognition of pregnancy (MRP), around Day 16 of pregnancy (Humblot, 2001; Diskin *et al.,* 2011). During this critical period, embryonic survival depends on a complex crosstalk between the early conceptus and the mother, still poorly understood (Forde *et al.*, 2011b). The presence of a viable conceptus determines the disruption of the luteolytic signal and maintenance of the corpus luteum (CL), providing adequate progesterone (P_4) concentrations to induce uterine receptivity (Niswender *et al*., 2000).

Maternal inadequate circulating concentrations of P_4 are thought to be the main cause of early embryonic loss (Diskin & Morris, 2008). Conversely, high circulating P_4 concentrations in the immediate post-conception period were associated with advancement in embryo development and higher pregnancy rates, both in cattle and in sheep (Lonergan, 2011b). However, the ability of the early embryo to produce P_4 and the influence of the early embryo on maternal P_4 production remains elusive. It is also unclear whether embryonic development and survival are a result of a direct interaction with the CL or if those events are mediated through uterine effects. Prostaglandins (PGs) are involved in several reproductive events, including luteal regression, ovulation, pregnancy and parturition (Weems *et al*., 2006). These mediators are produced by the CL, uterus and conceptus, but their precise role in early embryo-maternal interactions is also unclear.

Embryo-maternal crosstalk remains one of the most challenging subjects in reproductive biology. The deciphering of embryo-maternal interactions may allow the development of new therapeutic strategies to enhance embryonic survival. This would have a major impact in cattle reproductive efficiency and profitability of modern cattle industry. Additionally, in a species comparative approach, the investigation of the mechanisms of mammalian early development may provide relevant advancements in our knowledge of the determinants of normal and abnormal deviations of health.

The main objective of the work presented in this thesis was to evaluate the embryo-maternal steroidogenic and prostanoid interactions leading to embryonic development and survival in cattle. Additionally, therapeutic strategies to enhance *in vivo* embryonic survival were also evaluated. This thesis comprises three *in vitro* studies and two *in vivo* studies with the following specific objectives:

In vitro studies:

a) To develop a bovine luteal cells *in vitro* culture system suitable for co-culture with early embryos. This culture system attempts to mimic putative embryo-luteal interactions, ruling out effects of other maternal tissues such as the endometrium;
b) To evaluate putative direct steroidogenic and prostanoid interactions between bovine early embryos and luteal cells, using a novel *in vitro* embryo – luteal cells co-culture model;
c) To determine the ability of the early embryo to transcribe genes coding for enzymes of the P_4 and PGs (prostaglandin E_2 - PGE_2 and prostaglandin F_2 alpha - $PGF_{2\alpha}$) synthesis pathways, and to produce these substances. Additionally, the effects of oocyte donor age (prepubertal versus post-pubertal cyclic heifers) and embryonic stage of development on embryonic gene transcription and P_4 and PGs production were also evaluated.
d) To investigate the existence of an embryotrophic effect of luteal origin, and of a luteotrophic effect of embryonic origin, as putative relevant components of the early embryo-maternal crosstalk.

In vivo studies:

a) To develop two novel *in vivo* models to evaluate embryo-maternal interactions and therapeutic strategies to enhance embryonic survival: the first model considered a low developmental competence embryo plus a high fertility recipient (demi-embryo plus virgin dairy heifer); the second model considered a low developmental embryo plus a poor fertility recipient (demi-embryo plus high-yielding dairy cow). In these models, embryonic growth and survival, and maternal luteal and placental functions were assessed;

b) To evaluate treatments with human chorionic gonadotrophin (hCG) and carprofen at embryo transfer (ET), designed to enhance survival of compromised embryos;
c) To evaluate treatment with hCG at ET, designed to enhance survival of compromised embryos in a poor fertility recipient;
d) To investigate the relative effects of presence and location of CL, and maternal plasma P_4 concentrations on embryo survival;
e) To describe maternal profiles of plasma P_4 and pregnancy-specific protein B (PSPB) in single and twin demi-embryo pregnancies;
f) To determine the effect of conceptus number on embryonic growth and on maternal luteal and placental functions.

These studies were converted in five manuscripts submitted for publication in international refereed and indexed journals and constitute the five chapters of the experimental work of this thesis, as follows:

Chapter I

Torres, A.*, Batista M.*, Diniz, P., Mateus, L. & Lopes-da-Costa, L. (2012). Development of a bovine luteal cell *in vitro* culture system suitable for co-culture with early embryos. *In Vitro Cell Dev Biol Anim,* 48(9), 583-592. *doi: 10.1007/s11626-012-9552-6.*
*both authors contributed equally to this work

Chapter II

Torres, A., Batista M., Diniz, P., Mateus, L. & Lopes-da-Costa, L. (2013). Embryo – luteal cells co-culture: an *in vitro* model to evaluate steroidogenic and prostanoid bovine early embryo-maternal interactions. *In Vitro Cell Dev Biol Anim*, Jan 29. doi: 10.1007/s11626-012-9577-x. [Epub ahead of print]

Chapter III

Torres, A., Batista, M., Diniz, P., Silva, E., Mateus, L. & Lopes-da-Costa, L. (2012). Effects of oocyte donor age and embryonic stage of development on transcription of genes coding for enzymes of the prostaglandins and progesterone synthesis pathways in bovine *in vitro*-produced embryos. Submitted to *Zygote*.

Chapter IV

Torres, A, Chagas e Silva, J., Deloche, M.C., Humblot, P., Horta, A.E.M., Lopes-da-Costa, L. (2013). Secondary corpora lutea induced by hCG treatment enhanced demi-embryo survival in lactating high-yielding dairy cows. *Reprod Domest Anim*, Jan 16. *doi: 10.1111/rda.12138.* [Epub ahead of print]

Chapter V

Torres, A, Chagas e Silva, J., Diniz, P., Lopes-da-Costa, L. (2012). Evaluation of treatments with hCG and carprofen at embryo transfer in a demi-embryo and recipient virgin heifer. Accepted for publication in *Animal*.

LITERATURE REVIEW

1. Embryo-maternal interactions in early pregnancy

In mammals, and particularly in ruminants, pregnancy establishment is the result of a complex crosstalk between the mother and the developing embryo/fetus. The protagonists of this dialogue and the mechanisms subjacent to their interactions are discussed below.

1.1. Embryonic development

Hartman *et al.* (1931) first provided a description of bovine ovulated oocytes and two-cell stage embryos. Fifteen years later, a more detailed description of developmental stages, from the unfertilized oocyte to the blastocyst, was reported by Hamilton & Laing (1946). In the oviduct, oocyte and spermatozoa fuse to form the one-cell stage embryo – the zygote. This cell undergoes a series of mitosis, which results in increasing numbers of embryonic cells (blastomeres) without an increase in embryonic size, a process designated by cleavage. The first cell cycle is completed around 30-36 hours post insemination and the second cell cycle 10-12 hours later (Gordon, 1994). At around the 32-cell stage, between Days 5 and 6 post fertilization, compaction of blastomeres occurs, followed by cell polarization and formation of a fluid-filled central cavity, the blastocoel (Van Soom *et al.*, 1992). The latter event, designated blastulation, is the result of fluid transfer across the intercellular connections of outer blastomeres, promoted by ion transport through the Na^+/K^+ pump (Wrenzycki *et al.*, 2003). During subsequent cavitation embryonic cells differentiate into trophectoderm (TE) cells, that form a single layer surrounding the blastocoel, and inner cell mass (ICM) cells, resulting in the first cell differentiated embryonic stage – the blastocyst. All embryonic tissues, as well as part of the extra-embryonic membranes, will develop from ICM cells (Rossant & Lis, 1979). Cells of the TE exclusively contribute to the extra-embryonic components of the placenta (Rossant & Croy, 1985). Further increase in blastocoel size induces blastocyst expansion, thinning and rupture of the zona pellucida (ZP), with release of the embryonic mass, a process known as eclosion or hatching.

1.1.1. Activation of embryonic genome

The zygote and early cleavage-stage embryo is thought to be transcriptionally quiescent, cleaving under control of cytoplasmic maternally inherited mRNA molecules until genomic activation occurs. The transition from oogenetic to embryonic genomic activation (EGA) is called the maternal-to-embryonic transition (MET; Telford *et al.*, 1990) and allows further embryogenesis to become dependent on the expression of the embryonic genome (Kanka, 2003; Walser & Lipshitz, 2011). In the bovine, onset of MET is thought to occur at the 8- to 16-cell stage. However, it was suggested that the onset of MET may be controlled temporally (i.e., at a time after fertilization) rather than at a developmental stage. Minor transcriptional activity was detected as early as the pronuclear stage after *in vitro* fertilization, whereas sensitivity to the transcriptional inhibitor α-amanitin was first detected at the 2– to 5–cell stage, and became predominant following the 6– to 8-cell stage (reviewed by Kanka *et al.*, 2012). Using a suppression subtractive hybridization technique, Vigneault *et al.* (2009) produced a library of newly transcribed genes in bovine 8-cell stage embryos. This allowed the authors to identify more than 300 unique embryonic transcripts at the EGA and some transcripts at the 6– to 8-cell stage. A recent study identified a switch from Cullin 1-like variant 1 transcripts to Cullin 1 variant 3 transcripts at the late 8-cell stage (Vodickova-Kepkova *et al.*, 2011). Authors concluded that Cullin 1-like, variant 1 mRNA represents a maternal transcript that is gradually degraded after fertilization, while Cullin 1 variant 3 transcripts represent new embryonic mRNA synthesized beyond the 8-cell stage.

1.1.2. *In vitro* production of bovine embryos

The first successful *in vitro* production of embryos was reported by Shenk (1880 – cited by Gordon, 1994), who inseminated oocytes from rabbit and guinea pig and identified the first cleavage. First attempts of *in vitro* embryo production in the cow were reported in 1968 (Adams *et al.,* 1968; Sreenan *et al.*, 1968). However, the birth of a live calf following *in vitro* fertilization and ET at the 4-cell stage was only achieved in 1981 (Brackett *et al.*, 1982). Since the 1960s, several animal species, including rabbits, sheep, cattle and even chicken eggs, were used for *in vivo* culture of bovine embryos, mainly within the oviduct, which is thought to play an important role in early embryonic development (Gordon, 1994).

Initially, cattle embryos *in vitro* cultured from the first stages of development did not progress beyond the 8- to 16-cell stage, whereas only embryos beyond this stage could achieve normal

compaction; blastulation and hatching in culture (Wright & Bondioli, 1981). This blockage in *in vitro* development was later shown to be caused by inadequate culture conditions, and could be reversed by changes in the medium composition and co-culture with somatic cells (Gordon, 1994). The use of oviduct organ cultures to support mammalian *in vitro* embryo development was first reported in the mouse (Biggers *et al*., 1962). In cattle, successful *in vitro* culture until the blastocyst stage of development was first achieved through the co-culture with trophoblastic vesicles and oviduct epithelial cells co-culture (Camous *et al.*, 1984; Eyestone & First, 1989). A variety of somatic cells, including primary cultures of reproductive tissues like granulosa (Fabbri *et al*., 2000), cumulus (Goto *et al*., 1988) and endometrial cells (Soong *et al.*, 1998), and established cell lines like Vero (Huang *et al*., 1997) or BRL cells (Kubisch *et al.*, 2001), were also shown to allow successful embryo culture.

Although the mechanisms by which somatic cells improve early embryo development remain elusive, several embryotrophic effects were described during or following co-culture with somatic cells: faster cleavage, higher blastocyst rate, reduced cell fragmentation, increased blastocyst cell numbers, improved morphological quality grade, reduced apoptosis, enhanced hatching, maintenance of viability prior to transfer, increased hCG secretion, "rescue" of poor quality embryos or subjected to manipulation procedures, improved pregnancy rates and live births (reviewed by Orsi & Reischl, 2007). Putative mechanisms of somatic cells action include removal of deleterious components from the culture medium, protection against oxidative stress and modulation of medium physico-chemical properties, altogether termed 'negative conditioning'. Somatic cells may also secrete embryotrophic factors (termed 'positive conditioning'), including specific proteins and various growth factors, like leukaemia inhibitory factor (LIF), colony stimulating factors (e.g. granulocyte-macrophage-colony stimulating factor–GM-CSF), fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), haematopoietic stem cell factor, transforming growth factors (TGF)-α, β1 and β2, insulin-like growth factors (IGF)-I and -II, and their binding proteins IGFBP-1, -2, -3, -4 and -5, as well as polyamines (reviewed by Orsi & Reischl, 2007). These beneficial effects of co-culture with somatic cell acquire more relevance with increasing duration of co-culture (Wiemer *et al*., 1989; Lai *et al*., 1996).

Co-cultures can be used to study embryo-maternal interactions (Lee & Yeung, 2006; Pereira *et al*., 2006, 2009). However, co-culture systems should address the different and changing requirements of somatic cells and embryos throughout the culture period (Gardner, 1998). Media designed for embryo culture may not provide the specific requirements of somatic cells, with consequential loss of their *in vivo* morphological and functional properties (Reischl

et al., 1999). Conversely, media designed for somatic cells culture may not support normal embryonic development. Altogether, this may invalidate the use of co-culture systems to study physiologic early embryo-maternal interactions. Therefore, an *in vitro* maternal reproductive tissue (luteal cells, oviductal cells, uterine cells) co-culture system suitable for embryo culture needs to be developed.

Sanitary concerns and deleterious epigenetic effects induced by *in vitro* culture conditions (see below), led scientists to develop new media formulations capable of supporting *in vitro* embryo development in the absence of somatic cells co-culture. New media should mimic the histotroph produced by oviductal and uterine cells. Formulation of the synthetic oviductal fluid (SOF; Tervit *et al*., 1972), based on the composition of sheep oviductal fluid, allowed development until the blastocyst stage, without somatic cell support. Since then, media have been improved in order to satisfy embryo metabolic needs and avoid genic expression disturbances associated with the use of complex media (Purpera *et al*., 2009).

Supplementation of SOF with serum enhanced embryo development (McLaughlin *et al*., 1990; Lim *et al*., 1994). Serum contains proteins, vitamins, energy sources, lipids and growth factors, promoting compaction, blastulation and high embryo cell numbers (Gordon, 1994). However, media high serum content was linked to disturbed embryonic morphology, metabolism and gene expression, and was also associated to the large offspring syndrome (Van Wagtendonk-de Leeuw *et al*., 1998; Niemann &Wrenzycki, 2000; Purpera *et al*., 2009). These effects, as well as risks of contamination, led scientists to develop chemically, protein free, defined media. Unfortunately, a consensually reliable medium suitable for bovine *in vitro* embryo culture has not been fully established.

Oviductal and uterine intraluminal oxygen tensions were shown to be lower than the atmospheric in many species (Mastroianni & Jones, 1965; Mitchell & Yochim, 1968; Fischer & Bavister, 1993). Low oxygen (O_2) tensions (5% instead of the 20% typical of air) are beneficial to *in vitro* embryo culture (Thompson *et al*., 1990; Liu & Foote, 1995; Lonergan *et al*., 1999). Culture under a low O_2 tension atmosphere diminishes the need for co-culture, since one of the benefits of co-culture is the protection against oxidative stress (Watson *et al*., 1994, Rizos *et al*., 2001). However, somatic cells, particularly those from well vascularized tissues like luteal cells, usually require an atmospheric oxygen tension in co-culture systems (Orsi & Reischl, 2007; Corrêa *et al*., 2008; Vajta *et al*., 2010). It is a common practice in embryo cultures the overlaying of the culture medium with paraffin or mineral oil, in order to prevent pH alterations and dehydration. Oil overlaying of culture medium also allows culture in micro drops, which enhances development, compared to large volume medium culture (Fukui *et al*., 1996). However, oil can absorb lipid-soluble components of culture media

(Miller & Pursel, 1987) eventually restricting their effects on embryonic and somatic cells metabolism. Additionally, movement of these lipophilic substances towards the oil fraction results in erroneous measurements in culture medium.

Despite decades devoted to improvement of culture conditions, *in vitro* production still leads to lower embryonic developmental competence, compared to that achieved *in vivo* (Lonergan *et al*., 2006). This difference in developmental competence is translated in terms of morphology, cryotolerance, gene expression patterns, and pregnancy rate following transfer (Rizos *et al*., 2002a, 2002b). Transfer of *in vivo* derived zygotes (i.e., matured and fertilized *in vivo*) to *in vitro* culture results in a high blastocyst rate, but the resulting blastocysts tend to be of poor quality. In contrast, culturing *in vitro* derived zygotes (i.e., maturated and fertilized *in vitro*) *in vivo*, in the oviducts of the same or of different species, although not affecting the blastocyst rate, substantially improves blastocyst quality. These observations indicate that developmental competence, at least to the blastocyst stage, is controlled to a large extent by oocyte quality, while the post-fertilization environment mainly affects embryo quality (Lonergan, 2011a).

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1.1.3. Assessment of embryo quality

The ultimate test of embryo quality is the ability to establish a pregnancy, resulting in the birth of a healthy offspring. As this represents a very expensive and time consuming methodology, researchers have been trying to identify markers of embryonic quality. Morphology assessment is widely used for embryo selection prior to transfer. In the bovine, blastomeres are opaque because of lipid droplet accumulation, making it difficult to assess nuclear and nucleolar morphology. Linder and Wright (1983) developed a morphological classification system with four grades (excellent, good, fair and poor), which were associated to pregnancy rates. The International Embryo Transfer Society (IETS) standardized an embryo quality classification, based on morphological assessment (Stringfellow & Seidel, 1998). Features such as color of the blastomeres, extent of compaction, timing of blastocyst formation and expansion, and embryo diameter at hatching can be linked with embryo quality (Van Soom *et al*., 2003). The timing of the first cleavage division is indicative of the developmental potential of the early embryo (Van Soom *et al*., 1992, 1997; Grisart *et al*., 1994; Holm *et al*., 1998; Lonergan *et al*., 2000), the faster cleaving embryos being those that develop to blastocyst at a higher rate. Embryo cell numbers have also been used as an indicator of bovine embryo quality. Data clearly shows that total cell number and ICM

number are higher in good than in poor quality embryos (reviewed by Gordon, 1994). Differential staining of the ICM and TE cells allows the calculation of the ICM/TE cells ratio (Van Soom *et al*., 2001).

More recently, development of molecular biology tools, such as the polimerase chain reaction (PCR), Real Time-PCR and microarray analysis, significantly widened our knowledge on embryo developmental competence. Microarray technology allowed the comparison between the transcriptional profiles of *in vivo* and *in vitro* produced embryos (Vodickova-Kepkova *et al*., 2011). These powerful tools identified a plethora of genes that may potentially be regarded as markers of oocyte and embryo quality. Oocyte and embryo developmental competence is probably a quantitative trait, dependent on small transcriptional changes of many individual genes in a well-orchestrated pattern (Kanka *et al*., 2012). Certainly, many of these genes are still not identified. In a future scenario, we can envisage the construction of a micro-array like tool that gathers the complete assemble of genes predictive markers of oocyte and embryo quality. Nevertheless, some genes, like *gpx1* (Bermejo-Alvarez, *et al.*, 2010), *igf2r* (Liu *et al*., 1997; Young *et al*., 2001), *slc2a1* (Balasubramian *et al*., 2007), *ptgs2* (El-Sayed *et al*., 2006; Saint-Dizier *et al*., 2011), *plac8* and *akr1b1* (El-Sayed *et al*., 2006), *tp53* (Favetta *et al*., 2004) and *shc1shc* (Leroy *et al*., 2010), were already suggested to be linked to embryo quality.

1.2. Corpus luteum (CL)

The CL is a transient endocrine gland that develops from the follicle wall after ovulation (Reynolds & Redmer, 1999). Granulosa cells (inner layer of the follicle wall) differentiate into large luteal cells (LLC), which represent 30% of the steroidogenic cells of the mature CL. These LLC secrete about 70% of the P_4 in a constitutive way (unresponsive to LH or cAMP stimulation). Theca cells (outer layer of the follicle wall) differentiate into small luteal cells (SLC), which comprise 70% of the steroidogenic cells of the mature CL. These SLC secrete only 30% of the P_4 and, unlike LLC, require LH stimulation for maximal production (Farin *et al.*, 1989; Niswender *et al.*, 1994). The CL consists of steroidogenic luteal cells and non-steroidogenic cells, i.e. vascular endothelial cells, fibroblasts, pericytes and immune cells such as lymphocytes, leucocytes and macrophages (Lei *et al.*, 1991).

The initial growth rate of the CL is similar to that of a developing tumor, however growth ceases at mid-cycle and CL size remains relatively constant if pregnancy is established (Reynolds *et al.*, 1994). Increase in size is dependent on an increase in SLC number and on

growth of LLC (Niswender, 1994). Neo-angiogenesis occurring within the developing CL leads to a highly dense and perfused vascular network, so that nearly every steroidogenic cell is in contact with a capillary (Redmer & Reynolds, 1996). Growth of the CL is stimulated by the autocrine and paracrine action of steroids, eicosanoids, cytokines and other growth factors (Reynolds & Redmer 1999; Berisha & Schams 2005). In the absence of pregnancy, the CL undergoes functional and morphological regression, a process termed luteolysis, which allows for the resumption of a new ovarian cycle. Functional regression occurs before morphological regression (O'Shea & McCoy, 1988; McCracken *et al.*, 1999). At the 16^{th} day of the cycle, $PGF_{2\alpha}$ from uterine origin reaches the CL, induces a sharp decrease in P_4 production and involution of luteal tissue, resulting in the formation of the corpus albicans, rich in collagen and connective tissue.

1.2.1 Steroidogenesis

Cholesterol, the common precursor of all steroids, is mainly diet derived and transported to the ovaries by circulating lipoproteins (low density lipoproteins – LDL – and high density lipoproteins – HDL; Ohashi *et al.*, 1982). Under lipid deprivation, LC are also able to synthesize cholesterol from acetate (Cook *et al.*, 1967; Cook & Nalbandov, 1968). The uptake of LDL by LC occurs through receptor mediated endocitosis (Brown & Goldstein, 1986), while HDL uptake is typically accomplished by membrane-bound HDL binding proteins (Lestavel & Fruchart, 1994). There appears to be some species differences in their preference for LDL or HDL, but either source can be used by LC of most species (Niswender *et al.*, 2000).

Once inside the cell, cholesterol can be metabolized for steroidogenesis or sterified with long-chain fatty acids and stored as cholesterol esters inside lipid droplets. In the cytoplasm, free cholesterol is transported to the mitochondria through a mechanism that involves cytoskeletal elements and sterol carrier proteins (Niswender, 2002), and then from the outer to the inner mitochondrial membrane (Stone & Hechter, 1954). This transport of cholesterol appears to be the rate-limiting step in the biosynthesis of P_4, as well as the primary site of acute hormonal regulation (Wiltbank *et al.*, 1993). Steroidogenic acute regulatory protein (StAR) may bind cholesterol in the cytosol and transport it to the mitochondrial membrane. Phosphorylation of StAR by protein kinase A (PKA) stimulates cholesterol transport, whereas phosphorylation by protein kinase C (PKC) may inhibit this process. Peripheral-type benzodiazepine receptors (PBR) are involved in the transport from the outer to the inner mitochondrial membrane. The

natural ligand of PBR, endozepine, also appears to be critical in cholesterol transport regulation, as targeted deletion of this molecule reduces basal steroidogenesis (Niswender, 2002). Once in the mitochondrial matrix, cytochrome P450 complex ($P450_{scc}$), adrenodoxin and adrenodoxin reductase cleave the side chain of cholesterol to form pregnenolone (P_5) (Stone & Hechter, 1954). This steroid is then transported to the smooth endoplasmatic rethiculum, where 3-β-hydroxysteroid dehydrogenase/Δ^5, Δ^4 isomerase (3β-HSD) converts P_5 to P_4 (Hanukoglu, 1992), which is then thought to leave the cell by diffusion (Niswender *et al.*, 2002). Because LC store minimal amounts of steroids, the regulation of circulating concentrations of steroids is accomplished primarily by the level of steroid synthesis and metabolism (Bose *et al.*, 2002).

1.2.2. Utero-ovarian countercurrent transport mechanism

The anatomical connections between the arterial, venous and lymphatic circulations of the ovary, oviduct and uterus allow for the passive transfer of energy (heat) and substances from one to another, without entering the systemic circulation and organ metabolization (at the liver, kidneys or lungs). Early studies in sheep demonstrated that ligation or surgical separation of the main uterine vein ipsilateral to the CL-bearing ovary inhibited the cyclic regression of the CL (Barrett *et al*., 1971; Baird & Land, 1973). Later on, Ginther (1974) demonstrated that $PGF_{2\alpha}$ of endometrial origin reaches the CL through direct passive transfer from the uterine vein to the ovarian artery, a process named utero-ovarian countercurrent transport mechanism. Transfer of steroid hormones and peptides through this mechanism was demonstrated in several species (Einer-Jensen, 1988), and significant differences between the concentrations of steroids in ovarian arterial blood and systemic blood was reported (Hunter *et al.* 1983).

On the other hand, substances produced in the ovary and released through the ovarian venous flow may reach ovarian arterial blood (rebound), the oviduct and the tubaric portion of the uterus. In ruminants, an ovarian-uterine countercurrent transport mechanism is critical for P_4 of luteal origin reach high concentrations in the uterine horn ipsilateral to the CL-bearing ovary (Weems *et al.* 1988). A preferential transfer of ovarian steroids to the uterus was also demonstrated in women, during both the follicular and luteal phases (Cicinelli *et al.,* 2004). This ovarian-uterine countercurrent transport mechanism is regarded as a local (ipsilateral) regulatory mechanism, involving mainly steroids and peptides (Einer-Jensen & Hunter, 2005). This is supported by early experiments involving anastomosis of the uterine vein or the

ovarian artery from the pregnant to the nonpregnant uterine horn, which indicated that both luteolytic and luteoprotective mediators only act locally (Mapletoft *et al.*, 1975, 1976). Additionally, embryo/conceptus transfer and hysterectomy experiments in sheep indicated that the luteolytic and luteoprotective mechanisms do not act systemically, but are locally established between the uterus and the CL (Moor *et al.*, 1969, 1970).

1.3. Embryo-oviductal interactions

The oviduct is the siege of critical reproductive events, including oocyte maturation, sperm capacitation, fertilization and early embryonic development (Hunter, 1988; Gandolfi *et al.*, 1989; Ellington, 1991). The proximal part of the oviduct, referred to as the ampulla, is implicated in the transport of the oocyte-cumulus complex and in fertilisation, whereas the distal part, named the isthmus, is involved in sperm storage and embryo transport towards the uterus (Kolle *et al.*, 2009; Hunter, 2012). Disturbances in events occurring in the oviduct may lead to infertility (Hunter, 2012).

The transport of oocytes and embryos is thought to be mainly modulated by endocrine and autocrine/paracrine signals locally produced by the oocyte/embryo. In rats and hamsters, fertilized eggs reach the uterus a little earlier than unfertilized eggs, suggesting an influence of embryos on oviduct activity, in which platelet-activating factor may be involved (Velasquez *et al.*, 1995). In the mare, unfertilized oocytes remain and degenerate within the oviduct, while viable embryos progress to the uterus (Van Niekerk & Gerneke, 1966). In this species, the embryo enters the uterus 5 to 6 days following ovulation, when the morula or young blastocyst can secrete prostaglandins to stimulate local myosalpingeal activity (Weber *et al.*, 1991b). Estrogens and PGs such as $PGF_{2\alpha}$, increase oviductal smooth muscle contractility and the speed of embryonic transport (Lindblom *et al.*, 1978; Weber *et al.*, 1991a; Kissler *et al.*, 2004), whereas P_4 induces oviductal smooth muscle relaxation and reduces the speed of embryonic transport (Lindblom & Hamberger, 1980). Oocyte and embryo transport is also driven by ciliary beating at the oviductal epithelium (Lyons *et al.*, 2006). Digital videomicroscopic analysis established that, in cattle, the mechanisms of transport are different in the ampulla and the isthmus (Kölle *et al.*, 2009). In the ampulla, transport occur depth between the folds, while in the isthmus transport is straight and fast. Interestingly, at the ampulla, viable oocytes attach to the oviductal epithelium, while degenerated oocytes float in the oviductal lumen. These observations indicate that the oviduct is able to select viable oocytes. Also, the early embryo (24–48 h after fertilization) was able to modify local

oviductal vascularization, induce the formation of secretory cells, and regulate ciliary beating. Altogether, the above studies indicate that the early embryo has the ability to change the oviductal environment, which prompts for an early embryo-oviductal crosstalk.

1.4. Embryo-uterine interactions

1.4.1. Maternal recognition of pregnancy

Ruminants developed a unique mechanism to rescue the CL from luteolysis and maintain pregnancy (Wooding, 1992). In sheep, Martal *et al.* (1979) first demonstrated that CL maintenance resulted from the production of a proteinaceous factor present in the pre-implantation elongating conceptus. This factor, initially called trophoblastin and later ovine trophoblast protein 1 (oTP-1) (Godkin *et al.*, 1982), is now known as ovine interferon-tau (oIFN-τ) (Roberts *et al.*, 1999). In the bovine, a similar secretory product of the trophoblast cells of the bovine conceptus is known as bovine interferon-tau (bIFN-τ; Helmer *et al.*, 1987; Thatcher *et al.*, 1989; Roberts *et al.*, 1992). These trophoblastins were shown to be produced by the elongating blastocyst from Day 10 to 21-25, peaking around Days 14 to 16 (Spencer & Bazer, 2004). Around Day 16 of pregnancy, IFN-τ indirectly maintains the CL by attenuating (in cows; Meyer *et al.*, 1995) or altering (in ewes; Zarco *et al.*, 1988) the luteolytic pulses of uterine $PGF_{2\alpha}$. In the ewe, IFN-τ suppresses transcription of genes coding for oestrogen receptors (ER) and oxytocin receptors (OTXR) in the uterine epithelium. In the cow, IFN-τ suppresses transcription of genes coding for OTXR but has no effect in uterine ER (Mann *et al.*, 1999). Generation of uterine luteolytic episodes of $PGF_{2\alpha}$ requires luteal secretion of oxytocin (OTX) and the interaction of OTX of pituitary and ovarian origin at their receptors, located mainly on endometrial epithelial cells (Demmers *et al.*, 2001). Thus, the luteolytic mechanism requires a sequence of events involving positive feedback loops between the endometrium and the CL (McCracken *et al.*, 1999). The presence of a viable conceptus within the uterus diminishes, and in most cases, totally abolishes the pulsatile release of $PGF_{2\alpha}$ (Roberts *et al.*, 1999). It is still controversial whether IFN-τ acts only on the uterus or also at the systemic level. In sheep, Oliveira *et al.* (2008) and Bott *et al.* (2010) reported high levels of IFN-τ in the uterine venous blood on Day 15 of pregnancy, as well as an increase in transcription of IFN-stimulated genes in peripheral tissues (leukocytes, liver, and CL) of pregnant sheep and cows. However, others (Gotkin *et al.*, 1987; Lee *et al.*, 2012) reported that ^{125}I-labeled ovine IFN-τ infused intrauterine on Day 12 post-ovulation of nonpregnant ewes

was retained within the uterus, since IFN-τ is a relatively large protein (19 kDa) and it cannot be transferred to the ovary through the utero-ovarian pathway. Additionally, the above teams did not detect the presence of IFN-τ, analysed by Western-blott and enzyme-linked immunosorbent assay (ELISA), in the uterine vein, ovarian artery and CL of Days 12, 14 and 16 of both cyclic and pregnant animals.

In addition to its antiluteolytic actions, bIFN-τ alters the transcriptome of the endometrium, in order to enhance conceptus elongation and to establish uterine receptivity to implantation (reviewed by Spencer *et al.*, 2008). A recent study using RNAseq (Forde *et al.*, 2012) showed that, although IFN-τ is critical for MRP, there may be other relevant mechanisms in the embryo-maternal crosstalk leading to pregnancy establishment. Comparing the endometrium of pregnant and cyclic heifers on Day 16, several differentially expressed genes regulated by IFN-τ were identified. Expression of some of these genes in blood cells may be predictive of the pregnancy status of dairy cows as early as Day 18 following insemination. However, several other differentially transcribed genes are not directly regulated by IFN-τ *in vivo* and may therefore be regulated by yet unknown conceptus-derived factors.

Secretion of IFN-τ during early pregnancy is also relevant for maternal tolerance against the fetal allograft. Major histocompatibility complex (MHC) class I molecules, consisting of an alpha chain and a beta2-microglobulin (B2M), regulate immune rejection responses by discriminating self and non-self-proteins (Choi *et al.*, 2003). These molecules are increased by type I IFNs stimulation. In the ovine uterus, MHC class I alpha chain and B2M, although expressed primarily in endometrial luminal epithelium and glandular epithelium on Days 10 and 12 of the estrous cycle and pregnancy, were shown to have an increased expression restricted to endometrial stromal cells and glandular epithelium, on Days 14 to 20 of pregnancy. Also, intrauterine infusion of IFN-τ increased MHC class I and B2M expression in endometrial stromal cells and glandular epithelium, but not in luminal epithelium and superficial glandular epithelium. Silencing MHC class I alpha chain and B2M genes in endometrial luminal epithelium and superficial glandular epithelium during pregnancy may be critical in preventing immune rejection of the conceptus allograft (Bazer *et al.*, 2008).

1.4.2. Implantation and Placentation

During early pregnancy, the endometrium produces a complex mix of compounds, known as the histotroph, which is released into the uterine lumen. The histotroph contains nutrients such as amino acids and glucose, cytokines and other growth factors, fatty acids, transport proteins

for vitamins and minerals and enzymes (Bazer *et al.*, 2012). Studies using the sheep uterine gland knockout model demonstrated that functional uterine glands are essential for ewes to experience normal estrous cycles and to support conceptus development (Spencer *et al.*, 2004). The endometrium plays a central role in the context of early embryo-maternal crosstalk. This dialogue induces dynamic changes in the uterine epithelia, tightly regulated by steroid hormones, cytokines and growth factors, which establish uterine receptivity towards the developing conceptus (Spencer *et al.*, 2008).

Implantation may be invasive (interstitial or eccentric) or non-invasive (central) depending on whether the TE invades through the uterine luminal epithelia and superficial glandular epithelia into the stroma or not. Implantation in domestic animals is protracted and superficial, with conceptus TE only attaching to uterine luminal epithelia and superficial glandular epithelia. This protracted pre-implantation period allows large spherical conceptuses to migrate (horse) and hatched blastocysts to elongate (swine and ruminant species) before apposition and attachment to the endometrium, prior to implantation (Bazer *et al.*, 2009). In ruminants, a dialogue between the free-floating blastocyst and the receptive endometrium, initiates the process of implantation (Tabibzadeh & Babaknia, 1995). After hatching from the ZP, blastocysts start to elongate on Day 12 (sheep) or Day 15 (cattle) to filamentous conceptuses that occupy the entire length of the uterine horn (Spencer *et al.*, 2008). This elongation, which is not observed *in vitro* (Heyman *et al.,* 1984), is critical for production of IFN-τ, MRP and implantation (Spencer *et al.*, 2008).

In the cow, implantation is preceded by blastocyst elongation, and apposition, attachment, and adhesion of the TE to the endometrial luminal epithelium (Chavatte-Palmer & Guillomot, 2007). TE-derived cells form a single layer that covers the external surface of the placenta, in contact with the endometrium. Together with the somatic (parietal) mesoderm, they will constitute the chorion. The allantois, which is a sac-like structure that arises as an out-pocketing of the fetal hindgut, ultimately expands to fuse with the chorion, forming the chorioallantois, a richly vascularized membrane (Schlafer *et al.*, 2000). TE-derived cells are referred to as trophoblast cells, which include two morphologically and functionally distinct types: mononucleate and binucleate trophoblast cells (Igwebuike, 2006). Mononucleate cells comprise the majority of the interface and are primarily involved in nutrient exchange, although they are responsible for the production of the pregnancy recognition signal, IFN-τ (Farin *et al.*, 1990). Binucleate cells secrete into the maternal circulation hormones such as placental lactogen, P_4 and pregnancy-specific (-associated) proteins (PAGs) (Becker s*et al.*, 1998). These cells migrate across the fetal-maternal interface to fuse with endometrial epithelial columnar cells forming trinucleate hybrid cells. Continued binucleate cell migration

and fusion with trinucleate cells, results in the formation of syncytial plaques and gave rise to the classification of the ruminant placenta as synepitheliochorial. In the cow and deer syncytial plaques are present at implantation, but become displaced by overgrowth of endometrial epithelial cells, whereas in the sheep and goat the caruncular syncytial plaques continue as a consistent feature in the placentomes throughout pregnancy (reviewed by Igwebuike, 2006).

The placenta provides the developing fetus with nutrients, gas transport, immunological and physical protection, and waste product removal (Leiser & Kaufmann, 1994). In ruminant ungulates, the placenta is classified as cotyledonary on the basis of its gross anatomical features. It exhibits discrete areas of attachment, the placentomes, formed by the interaction of patches of the chorioallantois with the endometrium. The foetal portion of the placentome is the cotyledon, whereas the maternal contact sites are the caruncles (Igwebuike, 2006). A close interdigitation between the trophoblast cells of the foetal villi and endometrial epithelial cells ensures efficient fetal-to-maternal communication, even though the fetal cells are never in direct contact with maternal blood (Nguyen *et al.*, 2012).

1.4.3. Pregnancy associated glycoproteins

The PAGs family belongs to the aspartic proteinase superfamily (Xie *et al.*, 1991). At least 21 bovine PAGs were identified (Green *et al.*, 2000). They are abundantly expressed in the outer cell layer of the placenta of ruminants until parturition. These glycoproteins are divided in two groups. The group largely localized in the placental fetomaternal interface is considered of ancient origin and is designated as PAG2. A separate subgroup expressed primarily in trophoblastic binucleate cells, considered to have resulted from a more recent series of gene duplications, is designated PAG1. In cattle, some PAGs from this subgroup become detectable in the maternal circulation at around Day 25 of gestation, with concentrations steadily increasing throughout pregnancy and peaking just before parturition. Their function is thought to be related to immunomodulation, trophic support of the CL, and regulation of trophoblastic cell migration (Wooding *et al.*, 2005). Several molecules of the PAG family were isolated from the cotyledons of ewes, goats and cows. Some of these molecules were used to immunize rabbits and the antisera obtained allowed the development of homologous and heterologous RIA systems for PAG measurements in serum, plasma or milk samples (reviewed by Szenci *et al.*, 1998). Among these PAGs are bovine pregnancy-specific protein B (bPSPB), and bovine pregnancy-associated glycoprotein 1 (bPAG1). Because both bPSPB

and bPAG1 are found in the maternal circulation during pregnancy, these proteins are good indicators of the presence of a live embryo. Concentrations of these PAGs were also valuable for predicting fetoplacental viability (Patel *et al.*, 1995; 1998; Vasques *et al.*, 1995; Hashizume *et al.*, 2002).

Pregnancy-specific protein B was first detected in the bovine placenta in the early 80s (Butler *et al.*, 1982). This allowed the development and commercial application of an ELISA to detect the presence of PSPB as a biochemical marker of pregnancy (Sasser *et al.*, 1986, 1989). In the ewe, PSPB was shown to regulate luteal and placental PGE_2 secretion after Day 50 of pregnancy (Weems *et al.*, 1997, 2003a,b), which in turn regulates luteal P_4 (Weems *et al.*, 2003b). After parturition or abortion concentrations of PAGs remain in circulation for 2 – 3 months (Humblot *et al.*, 1988; Mialon *et al.*, 1993; de Sousa *et al.*, 2003). Therefore cows inseminated early in the postpartum period may have concentrations of PAGs above the pregnancy/open cut-off value, which may affect the precision of pregnancy diagnosis. Also, variation in serum PAGs concentrations among cows in early gestation preclude their reliable use at Days 26–30 of pregnancy (Zoli *et al.*, 1992; Humblot, 2001). Concentrations of PSPB throughout pregnancy differ between single and twin pregnancies in the cow (Dobson *et al.*, 1993; Patel *et al.*, 1995), ewe (Willard *et al.*, 1995) and goat (Humblot *et al.*, 1990). However, in *in vitro* produced embryos these differences between single and twin pregnancies were not consistent at all stages of gestation (Vasques *et al.*, 1995).

1.5. Progesterone

Most of known effects of P_4 are exerted through the regulation of gene transcription. This occurs upon coupling with specific nuclear receptors that act as ligand-inducible transcription factors (Nieswender *et al.*, 2000). Expression of these P_4 receptors (PRs) is induced by previous exposure to estrogens, which also downregulate expression of their own receptors (Evans *et al.*, 1980; Iwai *et al.*, 1995). There are two isotypes of PR, PRA and PRB (Lim *et al.*, 1999). After binding to receptors, a classical genomic pathway induces conformational changes that lead to dimerization of receptors and their activation, usually by phosphorylation. Dimmers are relocated inside the nucleus and transcription of target genes is activated (Conneely *et al.*, 2003). PRs were detected in the bovine uterus (Robinson *et al.,* 2001; Okumu *et al.*, 2010), CL (Rueda *et al.*, 2000) and blastocyst (Clemente *et al.*, 2009). Over the last years, it became evident that P_4 effects cannot be exclusively mediated by nuclear receptors, because activation of these receptors results in a considerable latency

period until the response is triggered. This contrasts with some effects of P_4 stimulation, which are established within few minutes (Falkenstein *et al.*, 2000). Therefore, it was proposed that P_4 and other steroid hormones can mediate non genomic effects by two ways: i) non specifically, changing membrane fluidity (not mediated by receptors); or ii) via specific steroid receptors, including modified classical nuclear receptors and non classical membrane associate P_4 receptors (Meyer *et al.*, 1996). There are at least three different proteins, which can be potential membrane P_4 receptors: membrane progestin receptor (mPR), RDA288 protein and P_4 membrane receptor component 1 (PGRMC1) (Peluso, 2006; Wehling *et al.*, 2007). The main targets of P_4 action are the reproductive tract, the mammary gland and the hypothalamo-pituitary axis. The most relevant effect of P_4 is to prepare the reproductive tract for establishment and maintenance of pregnancy.

In the ovary, studies using P_4 antagonists and PR knock-out mice led to the conclusion that PR is required specifically for LH-dependent follicular rupture leading to ovulation but not for differentiation of granulosa cells into luteal cells (reviewed by Conneely *et al.*, 2001). In the CL, P_4 was shown to stimulate its own production (Skarzynski & Okuda, 1999; Duras *et al.,* 2005), and affect transcription of genes encoding steroidogenic enzymes (Rekawiecki *et al.*, 2005). Blockage of autocrine and/or paracrine action of P_4 by a specific antagonist (onapristone) reduced secretions of OXT, $PGF_{2\alpha}$ and PGE_2 in early luteal cells, whereas in mid luteal cells, OXT secretion was reduced but $PGF_{2\alpha}$ secretion was stimulated (Skarzynski & Okuda, 1999; Okuda *et al.*, 2004). Therefore, P_4 stimulates P_4, OXT and PGs secretion in early luteal cells, but in mid-cycle luteal cells P_4 inhibits $PGF_{2\alpha}$ secretion. However, a positive autocrine regulation of P_4 was not observed in luteal cells of pregnant cattle (Weems *et al.*, 2002). Additionally, P_4 suppresses apoptosis in bovine luteal cells (Rueda *et al.*, 2000; Okuda *et al.*, 2004), stimulates transcription of anti-apoptotic bcl-2 mRNA on Days 6-15 of the cow estrous cycle, and decreases the ratio of bax/bcl-2 gene transcription (Liszewska *et al.*, 2005). *In vitro* blockage of P_4 action with onapristone was also shown to reduce luteal cells viability (Okuda *et al.*, 2004).

In the oviduct, P_4 receptors are expressed in the early luteal phase, both in the ampulla and the isthmus, in the luminal epithelium, stroma and muscle layer (Ulbrich *et al.*, 2003). These receptors include the typical nuclear receptors, and the membrane receptors PGRMC1 and its relative, PGRMC2 (Saint-Dizier *et al.*, 2012). In cattle, P_4 reduced oviduct motility *in vivo* (Bennett *et al.*, 1988) and ciliary motility in tubal explants *ex vivo* through rapid non genomic mechanisms (Wessel *et al.*, 2004), and quantitatively (Gerena & Killian, 1990) and qualitatively (Hugentobler *et al.*, 2010) changed secretion of growth factors.

In the uterus, P_4 acts in the endometrium and myometrium, promoting embryonic growth and development, MRP and uterine receptivity for implantation (Lonergan, 2011b). In the endometrium, P_4 actuates as a differentiation factor (Cummings & Yochim, 1984), inhibiting mitosis earlier promoted by estrogens in the follicular phase, inducing stromal differentiation, stimulating glandular secretions and changes in the pattern of proteins secreted by endometrial cells. In the myometrium, P_4 induces quiescence by increasing cell resting potential, preventing electrical coupling between myometrial cells, decreasing uptake of extracellular calcium that is required for contraction, and by blocking expression of estradiol-induced α-adrenergic receptors (Niswender *et al.*, 2000). Uterine receptivity to implantation is mediated by P_4 regulatory functions, either stimulating local production of several factors through autocrine and paracrine pathways, or inducing differential expression of PRs. In fact, uterine receptivity requires silencing of PRs expression in the luminal epithelium, superficial glandular epithelium and glandular epithelium, but continued expression of PRs in stromal cells and the myometrium (Bazer *et al.*, 2009). The effects of P_4 on PR-negative uterine epithelia are likely mediated through P_4 effects on PR-positive stromal cells, which stimulate expression of growth factors named 'progestamedins' (fibroblast growth factors-7 and -10 and hepatocyte growth factor). These growth factors will in turn exert paracrine effects on uterine epithelia and conceptus trophectoderm (Bazer *et al.*, 2008). Furthermore, early luteal high P_4 concentrations induce early downregulation of PR (Okumu *et al.*, 2010) and enhanced conceptus elongation (Forde *et al.*, 2009). In contrast, early luteal low P_4 concentrations induced a delay in the normal temporal pattern of endometrial gene expression, which was associated to a delay in downregulation of PRs in luminal and glandular epithelia, and a low ability to support conceptus development (Beltman *et al.*, 2009; Forde *et al.,* 2011a).

1.5.1. Progesterone and the early embryo

Several studies have reported a significant association between circulating low P_4 concentrations and low fertility. A delay in the rise of post-ovulatory P_4 concentrations was associated with retarded embryo growth and low secretion of IFN-τ on Day 16 post insemination (Mann & Lamming, 2001). Low P_4 was also associated with enhanced secretion of uterine $PGF_{2\alpha}$, leading to luteolysis and termination of pregnancy (Santos *et al.*, 2004). High-yielding lactating dairy cows have a high rate of steroid clearance, which may induce low luteal peripheral P_4 concentrations (Wiltbank *et al.*, 2006). Work from our team showed that lactating dairy cows have lower plasma P_4 concentrations than dairy heifers, and this is

associated to a lower embryo yield and quality following superovulation (Chagas e Silva *et al.*, 2002a) and a lower pregnancy rate following embryo transfer (Chagas e Silva *et al.*, 2002b). These observations are in agreement with other reports (Wiebold, 1988; Ryan *et al.*, 1993; Sartori *et al.*, 2002), comparing quality from embryos recovered from moderate- or high-producing lactating dairy cows, non-lactating cows and heifers. The former two groups of cows showed a higher proportion of embryos (41–67%) classified as abnormal, compared to the latter two groups (17–28%).

In the cow and ewe, P_4 supplementation in early pregnancy enhanced embryo development. Garrett *et al.* (1988) supplemented beef cows on Days 1, 2, 3, and 4 of pregnancy and obtained increased peripheral plasma P_4 concentrations from Days 2 to 5 and larger conceptuses on Day 14. Carter *et al.* (2008) supplemented P_4 on Days 3 to 8 and recovered larger conceptus at slaughter on Day 16. Satterfield *et al.* (2006), supplemented ewes with daily P_4 injections, starting at 36 h postmating, and observed an increased blastocyst diameter and an advanced blastocyst elongation on Day 9 and 12, respectively. These effects were blocked by administration of a PR antagonist. Treatment with hCG at Day 5, which induced secondary CL and high plasma concentrations of P_4 from Day 7 onwards, increased conceptus size at Day 14, which was highly correlated with enhanced IFN-τ secretion *in vitro* (Rizos *et al.*, 2009). Carter *et al.* (2010) transferred *in vitro* produced bovine zygotes to the oviducts of heifers with high or normal circulating P_4 concentrations. Although the level of circulating P_4 concentrations had no effect on the proportion of zygotes developing to the blastocyst stage, it induced subtle changes to the embryonic transcriptome that could be associated with advanced post-hatching elongation.

In an attempt to clarify whether P_4 has a direct effect on the embryo, P_4 was supplemented in *in vitro* embryo culture. Results have been contradictory, possibly because of addition of P_4 at different times of embryo development, oil overlaying of culture wells or co-culture with different cell types. Goff and Smith (1998) added steroid hormones (P_4 and estradiol - E_2) and found no effect on development to the blastocyst stage and on blastocyst size. Clemente *et al.* (2009) added increasing concentrations of P_4 to culture medium, both in the presence or absence of a BOEC monolayer, but found no effects on cleavage rate, blastocyst rate, blastocyst cell number and allocation, and relative transcript abundance of selected genes related to embryo quality. Similarly, Larson *et al.* (2011) supplemented culture medium with low and high concentrations of P_4 in two phases of culture (Day 1-3 and Day 4-7), and found no effects on cleavage and blastocyst rates. In contrast, Ghaemi *et al.* (2008) observed a significant positive effect of culture medium P_4 supplementation on mouse blastocyst development and Merlo *et al.* (2006) reported an improved embryo development from the 8-

cell to the blastocyst stage in a serum-free culture system, following medium P_4 supplementation. Finally, Ferguson *et al.* (2012) added P_4 to culture and observed a significant increase on embryo yield and an advanced kinetic of development. However, culture medium P_4 supplementation, with BOEC co-culture, induced impaired embryo development (Pereira *et al.*, 2009).

1.6. Prostaglandins (PGs)

Arachidonic acid (AA) is the primary precursor for the synthesis of PGs, a metabolic pathway that requires the serial action of several enzymes. Cytosolic phospholipase A_2 liberates AA from cell membrane phospholipids. Prostaglandin-endoperoxide synthases 1 (PTGS1) and 2 (PTGS2) (also known as cyclooxygenases 1 and 2; COX-1 and COX-2) then convert AA into PGH_2 (Smith & Dewitt, 1996). Prostaglandin F synthase (PGFS) converts PGH_2 into $PGF_{2\alpha}$. Alternatively, prostaglandin E synthases, such as PGES-1 (microsomal PGES-1), PGES-2 (microsomal PGES-2), and PGES-3 (cytosolic PGES) convert PGH_2 into PGE_2. $PGF_{2\alpha}$ and PGE_2 are transported competitively across cell membranes by solute carrier organic anion transporter family, member 2A1 (SLCO2A1) (also known as prostaglandin transporter; Schuster, 1998), which has equal affinity for both PGs (Banu *et al.*, 2003).

The effects of PGE_2 are mediated through binding with G-protein-coupled cell-surface receptors (Coleman *et al*., 1994). PGE_2 has four receptor subtypes: EP1, EP2, EP3, and EP4. These receptor subtypes are encoded by different genes and differ in their signal transduction pathways. EP1 activates the PKC and Ca^{2+} pathways. EP2 and EP4 activate the PKA pathway. Activation of EP3 produces a wide range of complex and opposite actions (Sugimoto *et al.*, 2000), Activation of EP1 and EP3 generally results in contraction of smooth muscle, while activation of EP2 and EP4 leads to its relaxation. Effects of $PGF_{2\alpha}$ are also mediated by a G-protein-coupled cell-surface receptor: FP. Activation of this receptor in turn activates the PKC and Ca^{2+} cell signaling pathways (Narumiya *et al.*, 1999).

PGs participate in the mechanisms of ovulation, luteal regression, implantation and maintenance of pregnancy, parturition and postpartum uterine involution (Weems *et al.*, 2006). PTGS2 (but not PTGS1) deficient mice showed disturbances in ovulation, fertilization, implantation, and decidualization, indicating that PGs play essential roles in these processes (Dinchuck *et al.*, 1995; Lim *et al.*, 1997; Davis *et al.*, 1999).

In the ovary, intra-follicular PGs content increases with follicle maturation, and indomethacin, an inhibitor of PG synthesis, abolishes LH-induced ovulation. This effect is reversed by

treatment with PGE_2 or $PGF_{2\alpha}$ (Murdoch *et al.*, 1993). In humans, inhibition of PTGS2 action was suggested as a potential contraceptive emergency measure. Studies in rabbits and women showed that PTGS2 inhibitors induced luteinized unruptured follicles, sometimes associated with a delay in the rise of P_4 and lower P_4 concentrations in the first half of the luteal phase (Killick & Elstein, 1987; Smith *et al.*, 1996; Pall *et al.*, 2001; Salhab *et al.*, 2001, 2003). However, although a recent study showed that treatment with celecoxib, a selective inhibitor of PTGS2 action, led to ovulatory dysfunctions, there were no significant differences in the rates of ovulation between treated women and controls (Edelman *et al.*, 2012). In cattle, $PGF_{2\alpha}$ treatment is involved in the ovulatory mechanism (Randel *et al.*, 1996; Bridges & Fortune, 2003). Treatment with $PGF_{2\alpha}$ induced ovulation in prepubertal heifers, and these ovulations were followed by a normal lifespan of the CL, which supports the hypothesis that $PGF_{2\alpha}$ induces ovulation by a luteolysis-independent mechanism (Leonardi *et al.*, 2012).

PGs and leucotrienes stimulate CL development and production of P_4 (Skarzynski *et al.*, 2008). *In vivo*, $PGF_{2\alpha}$ is considered to be the major luteolytic agent, whereas PGE_2 acts in a luteotropic and luteoprotective way, promoting the normal lifespan of the CL and P_4 production (Asselin *et al.*, 1996; Niswender *et al.*, 2000; Kotwica *et al.*, 2003). Receptors for PGE_2, PGI_2 and $PGF_{2\alpha}$ were described in both SLC and LLC (Chegini *et al.*, 1991). As discussed above, at the time of natural luteolysis, $PGF_{2\alpha}$ is released in a pulsatile manner by the endometrium. A minimum of five one hour-long pulses over a period of 48h is required to cause complete functional (decline in progesterone) as well as structural (loss of luteal cells) luteolysis in sheep (McCracken *et al.*, 2012). Systemic administration of $PGF_{2\alpha}$ during the mid-luteal phase of the estrous cycle increases luteal $PGF_{2\alpha}$ production in sheep. This increase can be inhibited by pretreatment with indomethacin in CL explant culture (Rexroad & Guthrie, 1979). Also, administration of a luteolytic dose of $PGF_{2\alpha}$ *in vivo* and treatment of luteal cells with $PGF_{2\alpha}$ *in vitro* induces expression of PTGS2 mRNA in luteal tissue or luteal cells (Tsai & Wiltbank, 1997, 1998).

Therefore, endometrial $PGF_{2\alpha}$ causes functional luteolysis and stimulate luteal synthesis of $PGF_{2\alpha}$, a relevant component of the positive feedback loop between the uterus and the CL during the process of structural luteolysis. However, although administration of $PGF_{2\alpha}$ in the cow during mid-luteal phase drastically reduces P_4 concentrations and the volume of the CL, the same dose of $PGF_{2\alpha}$ does not induce luteolysis during the early-luteal phase, until the fifth day of the estrous cycle (Henricks *et al.*, 1974). In this regard, some recent reports suggested that $PGF_{2\alpha}$ effects may be CL stage dependent, and may act as an antiluteolytic agent, rather than a luteolytic agent, in an early stage of development of the CL (Miyamoto *et al.*, 2010; Shirasuna *et al.*, 2010; Zalman *et al.*, 2012). In contrast, PGE_2 has been considered luteotropic

throughout the luteal stage, as high luteal concentrations of PGE_2 stimulate StAR, 3β-HSD and cytochrome $P450_{scc}$ gene expression and also increase their protein products, which lead to an increase in P_4 synthesis (Rekawiecki *et al.*, 2005). Also, intrauterine or intraovarian infusions of PGE_2 in nonpregnant ewes extend the inter-estrus interval and reduce luteal sensitivity to both endogenously secreted and exogenously administered $PGF_{2\alpha}$ (Pratt *et al.*, 1977; 1979; Reynolds *et al.*, 1981).

In the oviduct, *in vivo* experiments demonstrated that E-series PGs relax, while F-series PGs stimulate muscular activity of the oviduct, and that the response of oviductal muscle to PGs appears to be affected by ovarian steroids. P_4 increases the oviductal muscle response to PGE_2 and decreases the response to $PGF_{2\alpha}$ (Spilman & Harper, 1975; Szóstek *et al.*, 2011). Wijayagunawardane *et al.* (1998) showed that, in the bovine oviduct, during the luteal phase, the highest concentrations of oviductal P_4, PGE_2 and $PGF_{2\alpha}$ were observed in the oviduct ipsilateral to the CL-bearing ovary. Basal $PGF_{2\alpha}$ production was higher in the ampulla than in the isthmus, while the basal PGE_2 production was higher in the isthmus than in the ampulla (Siemieniuch *et al.*, 2009). Equine embryos secrete PGE_2 from as early as Day 5 after ovulation (Weber *et al.*, 1991a), and this hormone relaxes the smooth muscle of the isthmus to allow progression of the embryo into the uterus (Weber *et al.*, 1991a, 1995).

In the uterus, epithelial cells are responsible for most of the $PGF_{2\alpha}$ production, while stromal cells produce mainly PGE_2 (Fortier *et al.*, 1988; Asselin *et al.*, 1996). In epithelial cells, the basal production of PGE_2 and $PGF_{2\alpha}$ was significantly reduced by E_2 and significantly increased by P_4, whereas no significant effect of sex steroids on the basal production of PGs was detected in stromal cells. OXT stimulated the production of $PGF_{2\alpha}$ and PGE_2 in epithelial but not stromal cells, and treatment with E_2 significantly increased OXT-stimulated $PGF_{2\alpha}$ production in both epithelial and stromal cells and of OXT-stimulated PGE_2 production in epithelial cells (Asselin *et al.*, 1996).

Pregnancy changes the patterns of PGs production and sensitivity to PGs actions. In the ewe, the CL is more resistant to the luteolytic effect of $PGF_{2\alpha}$ on Days 12-16 of pregnancy, and this resistance to luteolysis is increased when multiple embryos are present (reviewed by Lee *et al.*, 2012). In bovine caruncular crypts, PTGS2 and EP2 receptors are increased and co-expressed during early pregnancy and may be regulated by INFs (Parent *et al.*, 2002; Arosh *et al.*, 2003). PGs production following IFN-τ stimulation has been the research focus of several teams. *In vitro* studies demonstrated that the effect of IFN-τ on expression of PGs synthesis enzymes is dose-dependent, small doses being inhibitory and larger doses being non-inhibitory or even stimulatory (Parent *et al.*, 2003; Guzeloglu *et al.*, 2004). Low-dose IFN-τ stimulation of bovine primary uterine epithelial cells inhibited basal $PGF_{2\alpha}$ secretion whereas

high doses stimulated $PGF_{2\alpha}$ secretion and induced a shift towards PGE_2 secretion (Asselin & Fortier, 2000). Also in bovine primary uterine epithelial cells, Parent *et al.* (2003) demonstrated that IFN-τ blocked OTX-increased expression of PTGS2 and secretion of $PGF_{2\alpha}$, and down-regulated OXTR at the transcriptional level (Horn *et al.*, 1998). In cows, Emond *et al.* (2004) demonstrated that uterine PTGS2 is upregulated in response to intrauterine infusions of IFN-τ. Also in cows, Arosh *et al.* (2004a) demonstrated that in the endometrium IFN-τ decreased PGFS expression in epithelial cells and increased EP2 expression in stroma, whereas in the myometrium it decreased PGFS expression and increased EP2 expression in smooth muscle cells. Although IFN-τ stimulation had no effect on PGES expression, the above changes led to an increase in the PGES to PGFS ratio in endometrium and myometrium. Finally, in uterine epithelial cells, IFN-τ stimulation shifted the primary PG product from $PGF_{2\alpha}$ to PGE_2, and in stromal cells, where PGE_2 is the primary PG product, production of both PGs were increased at the same level. PTGS2 expression was also upregulated in the endometrium by IFN-τ stimulation, whereas PTGS1expression was not affected (Asselin *et al.*, 1997; Arosh *et al*., 2004a, Emond *et al.*, 2004).

In the CL, IFN-τ increases PGES expression and decreases EP3 expression, which indicates that PGE_2 may also play a pivotal role in luteal maintenance mediated by IFN-τ (Arosh *et al.,* 2004a). More recently (Lee *et al.*, 2012) demonstrated, in the sheep model, that luteal PGs synthesis was selectively directed towards $PGF_{2\alpha}$ at luteolysis and towards PGE_2 during the establishment of pregnancy. Compared to pregnant ewes, non-pregnant females had a higher $PGF_{2\alpha}$ to PGE_2 ratio in ovarian venous plasma on Days 14 and 16 of the estrous cycle. In contrast, in pregnant ewes the PGE_2 to $PGF_{2\alpha}$ ratio is higher on Days 14 and 16 of pregnancy. In an *in vitro* experiment, Day 16 CL explants from cyclic ewes selectively convert PGH_2 to $PGF_{2\alpha}$. In contrast, Day 16 CL explants from pregnant ewes selectively convert PGH_2 to PGE_2. More interestingly, Lee *et al.*, 2012 also reported that Day 16 intrauterine concentrations of $PGF_{2\alpha}$ were significantly higher than those of PGE_2 on both cyclic and pregnant ewes. However, at luteolysis ~85% of $PGF_{2\alpha}$ was transported from the uterus to the uterine vein, while at pregnancy establishment only ~35% of $PGF_{2\alpha}$ was transported from the uterus to the uterine vein. From these observations, the above authors suggested that in ruminants $PGF_{2\alpha}$ is secreted and transported from the endometrium to the uterine vein (endocrine secretion) at luteolysis and from the endometrium to the uterine lumen (exocrine secretion) at pregnancy establishment.

1.6.1. Prostaglandins and the early embryo

PGs production by the blastocyst was detected in several species: bovine (Shemesh *et al.*, 1979), rabbit (Dey *et al.*, 1980), human (Holmes & Gordashko, 1980), ovine (Hyland *et al.*, 1982), pig (Davis *et al.*, 1983), equine (Watson & Sertich, 1989; Weber *et al.*, 1992) and mouse (Marshburn *et al.*, 1990). Shemesh *et al.* (1979) first reported $PGF_{2\alpha}$ and PGE_2 production from bovine blastocysts collected at Days 13, 15 and 16. Additionally, Hwang *et al.* (1988) reported bovine embryo production of PGE_2 before Day 12, both PGE_2 and $PGF_{2\alpha}$ between Days 13 and 15 and prostaciclin (PGI_2) after Day 15. Lewis, (1982) confirmed both PGE_2 and $PGF_{2\alpha}$ *in vitro* production by bovine conceptuses from Day 16 to Day 19. Wilson *et al.* (1992) reported that PGE_2 and PGI_2 production by the conceptus was already detectable in Day 10 bovine embryos.

Recently, Saint-Dizier *et al.* (2011) identified transcription of PGES in bovine blastocysts, and observed a significant positive association between PGES transcription with embryonic quality. Transcription of PTGS2 was previously identified in Day 8 to Day 17 ovine embryos (Charpigny *et al.*, 1997), in Days 12 and 14 equine embryos (Aurich & Budik, 2005), in human *in vitro*-fertilised morulae and blastocysts (Wang *et al.*, 2002), in murine morulae and blastocysts collected *in vivo* (Tan *et al.*, 2005) and in Day 7 bovine *in vitro* produced embryos (Clemente *et al.*, 2009; Saint-Dizier *et al.*, 2011). High PGE_2 secreted into culture media during bovine oocyte maturation was associated to a higher cleavage rate thereafter (Gurevich *et al.*, 1993), and *in vitro* culture medium supplementation with PGE_2 increased the hatching rate of ovine embryos (Sayre & Lewis, 1993). The role of $PGF_{2\alpha}$ in embryo development is more controversial. Several studies reported that *in vitro* culture medium supplementation with $PGF_{2\alpha}$ decreased blastocyst yield and hatching rate in the bovine (Fazio *et al.*, 1997; Scenna *et al.*, 2004), rabbit (Maurer & Beier, 1976), and rat (Buuck *et al.*, 1997) species. However, Soto *et al.* (2003) reported that $PGF_{2\alpha}$ inhibited bovine embryonic development only if supplementation was done during oocyte maturation or fertilization. In the goat, although supplementation with either PGE_2 or $PGF_{2\alpha}$ alone had no effect in embryo development, a high PGE_2 to $PGF_{2\alpha}$ ratio improved embryo hatching rate, indicating a role for of both PGs in this process (Sayre, 2007). Also, in Day 10 pig blastocysts, estrogens which are relevant in establishment of pregnancy in this species increased the PGE_2:$PGF_{2\alpha}$ ratio of embryo produced PGs (Rosenkrans *et al.*, 1992).

2. Embryonic mortality

Embryonic mortality is one of the major causes of reproductive failure and of economic loss in the dairy industry. The Committee on Bovine Reproductive Nomenclature (1972) defined the embryonic period as the portion of gestation from conception to the end of the differentiation stage, approximately at Day 42 of gestation. The fetal period was defined as the portion of gestation from Day 42 to the delivery of the calf. Pregnancy loss is more common during the embryonic period and becomes less frequent beyond Day 50. Early embryo mortality occurs between fertilization and Day 24 of gestation, late embryo mortality occurs between Days 25 and 45, while fetal mortality occurs after this and up to parturition (Committee on Bovine Reproductive Nomenclature, 1972). In moderate and high yielding dairy cows actual total embryonic and fetal mortality rates are approximately 40% and 56%, and calving rates are approximately 55% and 40%, respectively (Diskin & Morris, 2008). In dairy cattle, within 5–6 days after insemination, only 65% of fertilized oocytes resulted in viable embryos, and the rates of fertilization for parous lactating, parous nonlactating, and nulliparous cattle were 50.0%, 57.9%, and 71.9%, respectively. This pattern was similiar in beef cows, except that rates were generally higher and comparable between parous nonlactating cows and nulliparous heifers (Santos *et al.*, 2004).

Successful embryonic development in mammals proceeds through a stereotypical sequence of developmental key events that includes: 1) formation of the blastocyst, 2) implantation into the uterus, 3) modulation of maternal ovarian function and uterine blood flow to facilitate establishment of pregnancy, 4) formation of the placenta including an increase in its surface area to facilitate nutrient uptake, and 5) development of the heart and vascularization of both embryo and fetus to facilitate nutrient delivery (Cross, 2001). Any disruption in these critical steps can lead to pregnancy wastage.

Reproductive inefficiency due to either fertilization failure following insemination or pregnancy loss prior to maternal recognition of pregnancy and CL maintenance usually result in no alteration of the interestrus interval, whereas embryonic loss occurring around or after the period of CL maintenance may be indicated by extension of the interestrus or interovulatory interval. Humblot (2001) suggested that luteolysis and return to estrus prior to day 24 might be linked with early embryonic loss, whereas an interestrus interval >24 days could indicate embryonic losses after day 16.

The period of greatest reproductive wastage is thought to occur before Day 16 (Dune *et al.*, 2000; Diskin & Morris, 2008). Between Days 18 and 28, i.e. after MRP but before traditional methods of pregnancy diagnosis are widely used, embryonic mortality may be 5–10% (Fricke

et al., 1998; Tatcher *et al.*, 2001). An additional 5–10% embryonic mortality may occur in lactating dairy cows from Days 28 to 42 (late embryonic mortality; Santos *et al.*, 2004; Inskeep & Dailey, 2005). The incidence of late embryonic loss in dairy cows may vary widely and depend on milk production and heat stress. When evaluating embryonic losses in French dairy herds, Humblot (2001) reported a prevalence of late embryonic mortality of 14.7%. Late embryonic mortality after Day 27 was 3.2% in dairy cows producing 6000 to 8000 kg of milk per year in Ireland (Silke *et al.*, 2002). However, when high-producing dairy cows were subjected to heat stress, late embryonic loss occurred in 42.7% of cows (Cartmill *et al.*, 2001). Fetal death is typically less prevalent than early and late embryonic losses, however it can vary widely across geographical location, animal type, animal use, management, and age. Santos *et al.*, (2004) reported fetal losses of up to 11% in beef and dairy cows, whereas primigravid beef and dairy cattle had relatively low rates of fetal mortalities (2.5–4.2%).

Genetic causes of embryo death include chromosomal defects, individual genes and genetic interactions (Van-Raden & Miller, 2006). Chromossomal defects like translocations or lethal recessive conditions can be controlled through genetic testing programs directed to AI sires (reviewed by Diskin & Morris, 2008). Also, maternal inbreeding decreased by 2% the 56–70-day non-return rates per 10% inbreeding of the dam, whereas inbreeding of the embryo reduced by 1% the 70-day non-return rate for each 10% increase in the level of inbreeding of the embryo (Cassell *et al.*, 2003).

The use of reproductive technologies have been constrained by suboptimal efficiency in the production of embryos and an increase in embryonic and fetal survival associated with *in vitro* embryo production (Hansen & Block, 2004). Pregnancy rates following transfer of fresh IVP blastocysts are lower than those obtained following transfer of *in vivo* embryos (Kruip & den Daas, 1997; Rizos *et al.*, 2002a, 2002b; Farin *et al.*, 2006). Embryos produced by reproductive technologies can also lead to the condition known as large offspring syndrome (LOS). This syndrome was identified in sheep and cows, and is characterized by aberrant fetal and placental development, increased fetal myogenesis, dystocia, dysfunctional perinatal pulmonary activity, organomegaly, and increased mortality in early postnatal life (Horta, 1999; Fleming *et al.*, 2004).

In vitro embryo production allowed the use of pre-pubertal females as oocyte donors, reducing the generation interval and increasing the genetic gain (Lohuis, 1995). However, oocytes from pre-pubertal cattle have a lower developmental competence, compared to those originated from adult donors (Khatir *et al.*, 1996; Presicce *et al.*, 1997; Majerus *et al.*, 1999; Ptak *et al.*, 1999; Armstrong, 2001; Camargo *et al.*, 2005). Altered kinetics on development of pre-pubertal donor derived embryos was also reported (Ptak *et al.*, 1999; Majerus *et al.*, 2000;

Leoni *et al.*, 2006a), and associated to differential expression of developmentally important genes in cattle (Oropeza *et al.*, 2004) and sheep (Leoni *et al.*, 2006b). In the ovine, several studies reported a low *in vivo* survival of embryos originated from pre-pubertal donors (O'Brien *et al.,* 1997; Ptak *et al.*, 1999, 2003; Kelly *et al*,. 2005).

Cryopreservation is detrimental to embryonic survival. The two most widely used techniques of cryopreservation are controlled slow freezing and vitrification (Papadopoulos *et al.*, 2002). In the ovine, vitrification resulted in a decrease in pregnancy rate, this effect being more pronounced in the case of IVP embryos, compared with *in vivo* embryos (Papadopoulos *et al.*, 2002). Lactating cows receiving vitrified IVP embryos had a lower pregnancy rate than those receiving fresh IVP embryos, especially in summer (Chebel *et al.*, 2008). In beef cows, transfer of fresh embryos consistently produced higher pregnancy rates than transfer of frozen-thawed embryos (Spell *et al.*, 2001; Wallace *et al.*, 2010).

Advancement in reproductive technologies also allowed the bisection of embryos for production of monozygotic twins. Since the first reports of embryo bisection in cattle, this technique has been used to produce identical twins, facilitate embryo sexing and increase the number of transferable embryos (reviewed by Rho *et al.*, 1998). Half embryos, originated through bisection, roughly present half of the mass and of the cells of donor whole embryos, but the viability can be decreased after transfer because of cell damage and/or fall in cell number in comparison with intact embryos (Hashiyada *et al.*, 2005). The pregnancy rate of single half embryos is usually lower than that of whole embryos, although the pregnancy rate per embryo donor is not affected or is even improved (Lopes *et al.*, 2001). A comparison between whole and half *in vivo* produced embryos revealed that early and late embryonic mortalities of half embryos was also significantly higher than that of whole embryos, which was associated to a lower luteotrophic influence around Day 25 of gestation of half embryos, compared to whole embryos. Interestingly, fetal mortality, as well as embryo size on Day 42, was similar for both types of embryos (Chagas e Silva *et al.*, 2008; Lopes-da-Costa *et al.*, 2011).

Inadequate or abnormal luteal function occurs in different physiologic conditions such as puberty, resumption of cyclicity post-partum and transition to a new breeding season. Abnormal luteal function includes insufficient P_4 production, premature luteolysis or a persistent CL (Garverick & Smith, 1986; Hunter, 1991). In fact, a late rise in P_4 concentrations after ovulation or poor P_4 secretion during the luteal phase results in the development of poor quality embryos with low ability to produce IFN-τ at the critical time (Mann *et al.*, 1999). Low levels of P_4 in the previous cycle before AI are also connected with higher embryo loss,

probably because of premature oocyte maturation and subsequent low developmental competence (Diskin & Morris, 2008).

In lactating cows, a continuous high plane of nutrition appears to chronically elevate liver blood flow and the metabolic clearance rate of P_4 and E_2 (Sangsritavong *et al.*, 2002; Wiltbank *et al.*, 2006). Among the multifactorial causes of low fertility in lactating dairy cows, high feed intake associated with low concentrations of circulating steroids may contribute substantially to reduced embryo quality (Sartori *et al.*, 2010). In a study conducted by Rizos *et al.* (2010), IVP embryos were transferred to the oviducts of nulliparous Holstein-Friesian heifers and postpartum lactating Holstein-Friesian cows using endoscopy, and development to the blastocyst stage following recovery on Day 7 was evaluated. Embryo recovery rate was lower in cows (57%) than in heifers (79%) and, development to the blastocyst stage was also lower in cows (18%), compared to heifers (34%). This indicated that low circulating P_4 concentrations in postpartum dairy cows were associated with an impaired ability of the oviduct and uterus to support embryo development.

Another cause of embryonic mortality is endometritis, which is associated with impaired reproductive performance, due to reduced conception, reduced odds ratio for pregnancy over the breeding period and increased risk of reproductive culling. These effects can be mediated directly by bacterial products, such as lipopolysaccharide (LPS, endotoxin), or indirectly by inflammatory mediators, such as cytokines, eicosanoids, nitric oxide and oxidative stress, affecting both uterine and embryonic function. Embryos resulting from fertilization of oocytes exposed to LPS or $PGF_{2\alpha}$ during maturation, or from sperm subjected to oxidative stress, are less likely to develop to the blastocyst stage (Soto *et al.*, 2003; Hendricks & Hansen, 2010). Embryos exposed to inflammatory mediators during development have fewer TE cells than non-exposed embryos, whereas nitric oxide impairs embryo development and tumor necrosis factor-α (TNFα) increases blastomere apoptosis (reviewed by Gilbert, 2011). Extragenital inflammations, like mastitis, can also be harmful to embryonic development (Soto *et al.*, 2003).

3. Strategies to improve embryo survival

Several strategies have been designed to enhance embryo survival. Due to its unequivocal role in pregnancy establishment and maintenance, P_4 based strategies have received great attention from both researchers and practitioners. Strategies designed to increase post-ovulatory peripheral concentrations of P_4 include increasing the endogenous function of the primary CL,

inducing secondary CL, directly supplementing P_4, and nutritional manipulations either to decrease plasma concentrations of E_2 while increasing those of P_4, or to inhibiting the $PGF_{2\alpha}$-synthesizing enzymatic machinery in the endometrium during the critical period (Binelli *et al.*, 2001; Inskeep, 2004). Hormonal manipulations to increase P_4 include direct P_4 supplementation (Stevenson *et al.*, 2007), administration of gonadotrophin-releasing hormone (GnRH; Pursley & Martins, 2011), bovine somatotropin (BST; Moreira *et al.*, 2002), equine chorionic gonadotrophin (eCG; Bartolome *et al.*, 2012) and human chorionic gonadotrophin (hCG; De Rensis *et al.*, 2010). This latter hormone, as well as PGs inhibitors will be discussed in further detail in this section.

3.1. Human chorionic gonadotrophin (hCG)

In 1927, Ascheim and Zondek were the first to report that the blood and urine of pregnant women contained a gonad-stimulating substance, later designated as human chorionic gonadotrophin (hCG; Lunenfeld, 2004). This gonadotrophin is synthesized by the developing embryo from Day 2 to 8 after fertilization onwards and is later mainly a product of placental syncytiotrophoblast cells (de Medeiros & Norman, 2009).

hCG is used in domestic animals because it has a potent LH-like activity that lasts longer than the activity of LH itself. In dairy cows, plasma LH concentrations are markedly increased for 30 h after hCG administration and do not return to baseline concentrations until up 66 h (Schmitt *et al.*, 1996b). In cattle, treatment with hCG extends the life span of the CL, increasing endogenous P_4 synthesis, induces ovulation throughout the estrous cycle, promotes the formation of secondary CL and modifies follicular wave dynamics increasing the frequency of three-wave dominant follicular cycles (reviewed by De Rensis *et al.*, 2008; Rizos *et al.*, 2010). However, the repeated use of hCG in cattle induces a humoral immune response that can neutralize the hCG molecule and dramatically reduce binding to its receptor (Sundby & Torjesen, 1978). hCG stimulates differentiation of theca and granulosa cells into SLC and LLC, transformation of SLC into LLC, the increase in size of luteal cells (Farin *et al.*, 1988) and of the CL (Rajamahendran & Sianangama, 1992), which leads to an increase in the steroidogenic capacity of the primary CL (Fricke *et al.*, 1993; Schmitt *et al.*, 1996a; Diaz *et al.*, 1998). hCG given at the time of AI increases plasma P_4 concentrations from Days 5 to 12 of the estrous cycle (Helmer *et al.*, 1986; De Rensis *et al.*, 2008; Machado *et al.*, 2008).

hCG induces the formation of secondary CL through the ovulation and luteinization of responsive follicles (Rajamahendran & Sianangama, 1992). The administration of 1000 IU

hCG in postpartum beef cows induced luteinization irrespective of follicle size or dominance at the time of treatment (Cooper *et al.*, 1991), and even follicles smaller than 10 mm in diameter were able to form luteal tissue in response to hCG (Sheffel *et al.*, 1982). However, formation of secondary luteal structures was more efficient when hCG was given during the early luteal (Days 4 to 7) than during the follicular (Days 0 to 3) or midluteal (Days 8 to 12) phase of the estrous cycle (Helmer *et al.*, 1986; Price & Webb, 1989). hCG-induced secondary CL were smaller, persisted less time (Sianangama & Rajamahendran, 1996; Stevenson *et al.*, 2008) and produced less P_4 than the primary CL (Fricke *et al.*, 1993; Sianangama & Rajamahendran, 1996).

Although hCG increased P_4 concentrations in lactating dairy cows, dairy heifers, beef cows and beef heifers, the beneficial effects on pregnancy rate were controversial (Lonergan, 2011b). In some studies, treatment with hCG failed to significantly improve pregnancy rates in dairy heifers (Schmitt *et al.*, 1996b), dairy cows (Hansel *et al.*, 1976; Eduvie and Sequin, 1982; Helmer and Britt, 1986; Schmitt *et al.*, 1996b; Galvao *et al.*, 2006; Stevenson *et al.*, 2008), beef heifers (Morris *et al.*, 1976; Breuel *et al.*, 1990; Funston *et al.*, 2005) and beef cows (Hansel *et al.*, 1976). However, in other studies hCG treatment between Days 5 and 14 increased embryo survival and pregnancy rates in dairy cows (Rajamahendran & Sianangama, 1992; Santos *et al.*, 2001; Stevenson *et al.*, 2007), beef heifers (Breuel *et al.*, 1989; Rossetti *et al.*, 2011) and beef cows (Rossetti *et al.*, 2011). In beef cows treatment with hCG tended to increase pregnancy rates following AI at 5 of 6 locations (Dahlen *et al.*, 2010), and following ET at 2 of 3 locations (Wallace *et al.*, 2010). A further study on embryo transfer recipients reported a higher pregnancy rate in recipients treated with hCG on Day 6 (67.5%) than in control cows (45.0%) or cows receiving hCG on Day 1 (42.5%) (Nishigai *et al.*, 2002). Our team (Chagas e Silva & Lopes-da-Costa, 2005) previously reported that hCG-treated recipients with secondary CL had a higher Day 28 pregnancy rate than untreated heifers. However, this was observed in frozen-thawed ET-bred but not in AI-bred heifers, which prompted for a role of secondary CL in the rescue of compromised embryos.

Results of experiments evaluating the effect of hCG administration at breeding on conception rates are also equivocal. Increased conception rates in beef heifers (Brown *et al.*, 1973; Wagner *et al.*, 1973) and dairy cows (Rajamahendran & Sianangama, 1992) were observed but in other studies using lactating cows (Hansel *et al.*, 1979) or beef heifers (Breuel *et al.*, 1990), this effect was not reported. De Rensis *et al.* (2008) suggested that these differences could be related to the period of the year in which the treatment was performed, a positive effect occurring during the warm but not the cold period of the year. Recently, Rizos *et al.* (2012) tested the effect of hCG administration at Day 5, two days before ET, on conceptus

elongation on Day 14, and observed that hCG induced hypertrophy of the primary CL, formation of secondary CL, and increased P_4 concentrations from Day 7 onwards, which were associated to increased conceptus area and IFN-τ secretion *in vitro*.

3.2. Inhibition of luteolytic $PGF_{2\alpha}$

Inhibition of endometrial $PGF_{2\alpha}$-synthesizing pathways can be achieved by the administration of non-steroidal anti-inflammatory drugs (NSAIDs) like carprofen, flunixin meglumine or ibuprofen lysinate, which non-selectively inhibit the release of luteolytic and other pro-inflammatory prostanoids and cytokines. These drugs were tested at critical periods like AI, ET or onset of MRP, originating controversial results.

At AI, treatment with carprofen had no positive effect on pregnancy rate in dairy cows (Heuwieser *et al.*, 2011), although a rationale for treatment at this stage is difficult to ascertain. Manipulation of the uterus at ET may induce release of $PGF_{2\alpha}$ (Rowe *et al.*, 1980), which may be deleterious to luteal function and embryo survival. Treatment with ibuprofen lysinate one hour before ET increased pregnancy rate by 26% (Elli *et al.*, 2001), whereas flunixin meglumine administered at ET increased pregnancy rates although with different intensity, depending on location (5-32%) (Purcell *et al.*, 2005). Increase in pregnancy rate following flunixin meglumine treatment at ET was also dependent on embryo quality (Scenna *et al.*, 2005), or was not observed (McNaughtan *et al.*, 2002). At the onset of MRP (Days 13 to 16), FM administration also originated controversial results, some studies reporting increased pregnancy rates associated to treatment (Guzeloglu *et al.*, 2007; Merril *et al.*, 2007) but others observing no effect on pregnancy rates (Geary *et al.*, 2010; von Krueger & Heuwiesier, 2010; Rabaglino *et al.*, 2010; Rossetti *et al.*, 2011). In contrast, administration of meloxicam, a highly selective inhibitor of PTGS2 with a long acting effect, at MRP was detrimental to pregnancy rate (Erdem & Guzeloglu, 2010).

EXPERIMENTAL WORK

Chapter I - Development of a bovine luteal cell *in vitro* culture system suitable for co-culture with early embryos

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1 - Abstract

The crosstalk cross talk between the corpus luteum (CL) and the early embryo, potentially relevant to pregnancy establishment, is difficult to evaluate in the *in vivo* bovine model. *In vitro* co-culture of bovine luteal cells and early embryos (Days 2–8 post *in vitro* fertilization) may allow the deciphering of this poorly understood cross talk. However, early embryos and somatic cells require different *in vitro* culture conditions. The objective of this study was to develop a bovine luteal cell *in vitro* culture system suitable for co-culture with early embryos in order to evaluate their putative steroidogenic and prostanoid interactions. The corpora lutea of the different stages of the estrous cycle (early, mid, and late) were recovered postmortem and enriched luteal cell populations were obtained. In experiments 1 and 2, the effects of CL stage, culture medium (TCM, DMEM-F12, or SOF), serum concentration (5 or 10%), atmosphere oxygen tension (5 or 20%), and refreshment of the medium on the ability of luteal cells to produce progesterone (P_4) were evaluated. The production of P_4 was significantly increased in early CL cultures, and luteal cells adapted well to simple media (SOF), low serum concentrations (5%), and oxygen tensions (5%). In experiment 3, previous luteal cell cryopreservation did not affect the production of P_4, $PGF_{2\alpha}$, and PGE_2, compared to fresh cell cultures.

This enables the use of pools of frozen–thawed cells to decrease the variation in cell function associated with primary cell cultures. In experiment 4, mineral oil overlaying culture wells resulted in a 50-fold decrease of the P_4 quantified in the medium, but had no effect on $PGF_{2\alpha}$ and PGE_2 quantification. In conclusion, a luteal cell *in vitro* culture system suitable for the 5 day long co-culture with early embryos was developed.

Keywords: Luteal cells, Progesterone, Prostaglandins, Embryo, Bovine

2 – Introduction

In bovine species, events leading to pregnancy establishment rely on a complex cross talk between the mother and the early embryo, which is still very poorly understood (Forde *et al*., 2011b). This cross talk mainly involves three protagonists: the embryo (blastocyst), the uterus (endometrium), and the corpus luteum, (CL). It is known that progesterone (P_4), secreted by luteal cells, regulates the growth and elongation of the blastocyst and the secretion of interferon-tau by trophoectoderm embryonic cells, which in turn inhibits the regression of the CL (luteolysis) and maintains adequate P_4 concentrations to allow pregnancy establishment (Mann & Lamming, 2001). This is referred to as the maternal recognition of pregnancy mechanism, which initiates around Day 16 of pregnancy. The effect of P_4 on the embryo seems to be indirect, through changes in the endometrial transcriptome and composition of the histotroph (Lonergan, 2011b), although a direct effect was also reported (Merlo *et al*., 2006; Ferguson *et al*., 2012).

Luteal cells also produce prostaglandins (including $PGF_{2\alpha}$ and PGE_2; Rodgers *et al*. 1988; Hu *et al*., 1990), and it was recently reported in the sheep model that luteal synthesis of prostaglandins are shifted toward $PGF_{2\alpha}$ during luteolysis and to PGE_2 during early pregnancy (Lee *et al*., 2012). This mediator (PGE_2) is also produced by pre-eclosion (Saint-Dizier *et al*., 2011) and elongated bovine embryos (Shemesh *et al*., 1979; Wilson *et al*., 1992). These steroids and prostanoids of luteal origin can reach the uterus through systemic circulation or through a local countercurrent mechanism, directly from the ovarian vein and lymphatics to the uterine artery, while PGE_2 of embryonic origin may reach the ovary through an inverse countercurrent mechanism (Einer-Jensen & Hunter, 2005).

Little is known about the interactions between the CL and the embryo that may occur before the maternal recognition of pregnancy. *In vivo* steroid and prostanoid evaluation of embryo–luteal interactions is a difficult task due to extensive hormonal background noise and animal welfare implications. *In vitro* co-culture systems allow the study of interactions between different cell types (Orsi & Reischl, 2007; Pereira *et al*., 2009), and the development of an *in vitro* embryo–luteal cell co-culture system could provide relevant novel data regarding those interactions. However, co-culture of early embryos and luteal cells might not be easily achieved since embryos and luteal cells have different *in vitro* culture requirements, which are potentially conflicting. Unfavorable *in vitro* cultural conditions impair embryo development and promote epigenetic alterations (Rizos *et al*., 2002a; Lonergan *et al*., 2003; Purpera *et al*., 2009). Therefore, an embryo–luteal *in vitro* co-culture system should address embryonic requirements, although not significantly jeopardizing luteal cell function.

Most studies reporting the use of *in vitro* luteal cell cultures use short-time cultures (1–2 days; Skarzynski *et al*., 2007), complex media (Bowolaksono *et al.*, 2008; Jiang *et al.*, 2011), high concentrations of serum (Goyeneche *et al*., 2006; Skarzynski *et al*., 2007), atmospheric oxygen tensions (Nishimura *et al*., 2008), and a 37°C incubation temperature (Freshney, 2005). All these conditions are unsuitable for early embryo culture, which require long-time cultures (5–6 days), simple media such as "synthetic oviduct fluid" (SOF) (Holm *et al*., 1999), serum concentrations only up to 5% (Holm *et al*., 1999; Rizos *et al*., 2003), a hypoxic atmosphere (5%; Yuan *et al*., 2003), and a 38.5°C to 39°C incubation temperature (Wang *et al.,* 1991), to mimic luminal oviductal and uterine conditions (Fischer & Bavister, 1993).

Established bovine luteal cell lines are not readily available. To avoid variation due to the use of primary cell cultures from a single CL, pools of CL of similar stage and replicates within each experiment are necessary. The cryopreservation of luteal cells from pools of selected CL could potentially originate uniform material to use within an experiment or across several experiments. However, to the best knowledge of authors, the *in vitro* culture of cryopreserved luteal cells was not reported.

Embryos are usually cultured under a mineral oil overlay to prevent evaporation; however, this may potentially affect the measurement of lipophilic substances in the culture medium as these compounds may move toward the oil fraction. This was shown to occur with steroids like P4 and estradiol, but information regarding prostaglandins is scarce (Miller & Pursei, 1987).

Although embryo co-culture with somatic cells was extensively used in the early days of *in vitro* embryo production (Imahie *et al*., 1992), the use of luteal tissue was only reported in a short-term culture (Thibodeaux *et al*., 1994). These authors co-cultured single Day 8 bovine blastocysts with bovine CL explants for 24 h and observed a luteotrophic effect of embryos, resulting in an increase in P_4 production by luteal cells. This observation indicated an early communication between the blastocyst and luteal cells. The luteotrophic effect of early embryos was also observed upon other P_4–producing cells, the follicle granulosa cells (Vasques *et al*., 1998; Pereira *et al*., 2006).

The objective of this study was to develop a bovine luteal cell *in vitro* culture system suitable for co-culture with early embryos in order to evaluate their putative steroidogenic and prostanoid interactions relevant for pregnancy establishment.

3 - Material and Methods

3.1 – Media composition

All reagents and media were supplied by Sigma-Aldrich Química, S.A. (Madrid, Spain), except otherwise stated. Three media were compared: (1) TCM-199 (TCM; ref. M2154); (2) DMEM-F12 (ref 11039-021; Gibco, Invitrogen, Life Technologies, S.A., Madrid, Spain); and (3) SOF, which was manufactured in the lab according to the composition described by Holm *et al*. (1999). Media TCM and DMEM-F12 were supplemented with 2.5 µg/mL amphotericin b (ref. A2942), 0.05 mg/mL gentamicin (ref. G1522), 0.1% BSA (ref. G1522), and 1 µL/mL ITS (ref. I3146). Medium SOF was supplemented with amino acids (BME, ref. B6766, 30 µL/mL; MEM, ref. M7145, 10 µL/mL), 1 µL/mL ITS, 0.34 mM trisodium citrate (ref. 6448.1000; Merck, VWR, Carnaxide, Portugal), 2.77 mM myoinositol (ref. I7508), and 0.05 mg/mL gentamycin. Fetal calf serum (FCS; ref. 26140-079, Gibco) was added to the three media in a final 5 or 10% (*v*/*v*) concentration.

3.2 – Isolation of enriched luteal cell populations

The CL were collected postmortem at the local abattoir, from crossbred heifers (12–16 months old), and transported to the laboratory in ice-cold PBS within 1 h. The CL were classified as early (Day 1–4 post-ovulation), mid (Day 5–10 post-ovulation), and late (Day 11–17 post-ovulation) according to the criteria established by Ireland *et al*. (1980) and Miyamoto *et al*. (2000). Luteal tissue was mechanically separated from its fibrous capsule, chopped into small pieces, and further dissociated in a 0.2% collagenase (ref. 10103578001; Roche, Sistemas de Diagnóstico Lda., Amadora, Portugal) plus 0.1% DNase (ref. D5025) DMEM-F12 solution. The cell suspension was washed and centrifuged for 20 min at 400×g in a discontinuous Percoll (ref. P4937) gradient with 54, 45, 27, and 18% layers (Fridén *et al*., 1999). Cells recovered from the 18–27 and 27–45% interfaces were then successively centrifuged for 5 min at 400×g, 200×g, and 56×g in order to selectively recover the heaviest steroidogenic cells. The mean purity of these enriched luteal cell populations was 90% based on the detection of 3βHSD enzymatic activity (Redmer *et al*., 1991), and morphological evaluation showed that cell suspensions consisted of about 70 % small luteal cells, 20% large

luteal cells, 10% endothelial cells, and no erythrocytes. The viability of these enriched luteal cell suspensions was over 80%, as assessed by the Trypan Blue exclusion method.

3.3 – Experiment 1

The main effects CL stage (early versus mid versus late) and culture medium (TCM versus DMEM-F12 versus SOF) and their interactions were evaluated in a 3×3 factorial design. Refreshment of the culture medium (no refreshment versus refreshment at Day 3 of culture) was also evaluated in a 3×3×2 factorial design. The production of P_4 by luteal cells in culture (as an indicator of main cellular function) was chosen as the end point measured variable. Measurements were done at Day 0 (culture seeding), Day 3 (accumulated production of Days 0–3), and Day 5 (accumulated production of Days 3–5), the medium being refreshed at sample collection on Day 3. Also, measurement was done at Day 5 in wells not refreshed (accumulated production of Days 0–5), in identical number to that of the refreshed wells. Replicates (n=5) of pools of six CL of each stage were used. Cell suspensions were seeded at 10^5 viable cells/well in 48-well culture multi-dishes (ref. 150687; Nunclon, Nunc, Roskilde, Denmark) in DMEM-F12 supplemented with 10 % FCS and incubated in a humidified atmosphere of 5% CO_2 in air at 39°C for 72 h. The medium was then replaced either by TCM, DMEM-F12, or SOF, supplemented with 10% FCS (day 0), the wells overlaid with 400 µL mineral oil (ref. M5310), and incubated in a humidified atmosphere of 5% CO_2+5% O_2 at 39°C for 5 days. Medium samples collected at Days 0, 3, and 5 were frozen and stored at –20°C until assay.

3.4 – Experiment 2

The main effects CL stage (early versus mid), culture medium (DMEM-F12 versus SOF), the concentrations of serum in the culture medium (5 versus 10%), oxygen tension in the culture atmosphere (5 versus 20%), and their interactions were evaluated in a 2×2×2×2 design with repeated measures (five levels corresponding to Days 1–5). Refreshment of the culture medium (daily refreshment versus no refreshment) was evaluated in a 2×2×2×2×2 factorial design. The production of P_4 by luteal cells in culture (as an indicator of main cellular function) was chosen as the end point measured variable. Measurements were done at Day 0

(culture seeding) and daily until Day 5 (Days 1–5). In this way, the value of Day 1 represents the accumulated production of Days 0–1 and so on. Also, measurement was done at Day 5 in wells not refreshed in identical number to that of the refreshed wells. Therefore, the effect of refreshment of the culture medium was evaluated comparing the sum of P_4 concentrations of Days 0–5 (daily refreshed wells) with the accumulated production of Days 0–5 (non-refreshed wells). Replicates (n=6) of each CL stage (single CL) were used to obtain enriched luteal cell suspensions, which were seeded at 10^5 viable cells/well in 24-well culture multi-dishes (ref. 142475; Nunclon) in DMEM-F12 plus 10 % FCS and incubated in a humidified atmosphere of 236 5 % CO_2 in air at 39°C for 24 h. The medium was then replaced (Day 0) either with DMEM-F12 or SOF, each supplemented either with 5 or 10% FCS. Half the wells were incubated in a humidified atmosphere of 5% CO_2 in air and the other half in a humidified atmosphere of 5% CO_2+5% O_2 for 5 days at 39°C. Medium samples were frozen and stored at −20°C until assay.

3.5 – Experiment 3

The effect of the cryopreservation of luteal cells on subsequent *in vitro* production of P_4, $PGF_{2\alpha}$, and PGE_2 (as indicators of the main steroidogenic and prostanoid functions) was evaluated. Replicates (n=12) of pools of two early-stage CL were used to obtain enriched luteal cell suspensions. Each cell suspension (4.3×106–6.9×10^6cells, 5 mL) in DMEM-F12 plus 10% FCS was plated in 25-cm^2 culture flasks (ref. 163371; Nunclon) and incubated in a humidified atmosphere of 5 % CO_2 in air at 39°C until 90% confluence. Cells were then trypsinized and half the cell suspension frozen in liquid nitrogen in DMEM-F12 plus 10% FCS with 10 %DMSO (ref. D2650), the other half of the cell suspension being seeded in a new 25-cm^2 culture flask. At 90% confluence of this culture, cells were trypsinized and their counterparts thawed. Frozen cells were kept submerged in liquid nitrogen for 24–48 h, depending on the time necessary for attainment of 90% confluence of the fresh cell counterparts. Both cell suspensions were seeded at 2×10^5cells/well in 48-well culture multi-dishes and incubated in a humidified atmosphere of 5%CO_2 in air at 39°C for 48 h, the medium being refreshed at 24 h of culture. Medium samples were collected at 48 h after seeding, frozen, and stored at −20°C (for P_4 measurement) or −80°C (for prostaglandin measurement). To evaluate cell number and viability at the end point of culture (48 h), fresh and frozen–thawed cell suspensions were seeded at 10^4cells/well in 96-well culture plates (ref. 167008; Nunclon) and incubated in the same conditions as described above. Following

culture, cells were either trypsinized and counted in a Neubauer chamber or submitted to a cell proliferation assay (CellTiter 96, AQueous One Solution Cell Proliferation Assay, Promega Corporation,Madison,WI) according to the manufacturer's instructions.

3.6 – Experiment 4

The effect of oil overlaying culture wells on P4, PGF2α, and PGE2 quantification in the culture medium was evaluated. Replicates (n=3) of pools of eight early-stage CL were processed, seeded in culture flasks, and frozen in liquid nitrogen as described above. Cells were thawed and seeded at 2.5×10^4viable cells/well in four-well culture dishes (ref. 144444; Nunclon) in DMEM-F12 plus 10% FCS and incubated in a humidified atmosphere of 5% CO^2 in air at 39°C for 24 h. The medium was replaced by SOF plus 5% FCS, half of the wells covered with 400 μL mineral oil, and the dishes incubated in a humidified atmosphere of 5% CO_2+5%O_2 at 39°C for 5 d. Medium samples were collected at Day 5, frozen, and stored at −20°C (for P_4 measurement) and −80°C (for PGs measurement).

3.7 – Measurement of P_4 concentrations

The concentrations of P_4 were measured by a solid phase RIA assay using commercial kits (Coat-a-Count, Siemens Healthcare Diagnostics, GmbH, Eschborn, Germany). The samples were assayed in duplicate. Intra- and inter-assay coefficients of variation were calculated according Rodbard *et al*. (1971). The inter-assay coefficients of variation were 8.45, 9.7, and 10.7% for three different controls used in the assay (for concentrations of 1.9, 3.7, and 17.4 ng/mL, respectively; Multivalent Control Module, Siemens Healthcare Diagnostics). The intra-assay coefficient of variation was 8.3%.

3.8 – Measurement of $PGF_{2\alpha}$ and PGE_2 concentrations

Medium samples were collected into tubes containing a stabilizer (0.3 M EDTA (ref. E5134) plus 1% aspirin (ref. A2093), pH 7.4). The PGE_2 concentrations in the culture medium were determined using a commercial enzyme immunoassay kit (PGE_2 Express EIA kit no. 500141, Cayman Chemical, Cayman Europe, Tallinn, Estonia). The $PGF_{2\alpha}$ concentrations in the

culture medium were measured by an enzyme immunoassay method as described previously (Skarzynski & Okuda, 1999). The coefficients of intra-assay and inter- assay variations were 14.3 and 2.5 % for the PGE_2 assay and 12.5 and 7.0% for the $PGF_{2\alpha}$ assay, respectively.

3.9 – Statistical analysis

The data were analyzed through the statistical software Statistica7® (Statsoft, 2004, Tulsa, OK). As the data did not follow a normal distribution, values were log-transformed for further analysis. In experiment 1, the main effects on the variable measured (concentrations of P_4) were analyzed by factorial ANOVA, while those in experiment 2 were analyzed by ANOVA with repeated measures and factorial ANOVA (effect of medium refreshment). In experiments 3 and 4, the main effect on the variables measured (concentrations of P_4, $PGF_{2\alpha}$, and PGE_2) was analyzed by single ANOVA. Significant effects were further analyzed post hoc by the Fisher LSD test. Significance was tested at the 5% level ($P<0.05$). In experiments 3 and 4, regression analysis between the concentrations of P_4, $PGF_{2\alpha}$ and PGE_2 was computed.

4 – Results

4.1 – Experiment 1

The main effects CL stage and culture medium significantly affected P_4 production in culture ($P< 0.00001$), while their interaction was not significant. As this interaction was non-significant, the data were pooled to illustrate each of the significant main effects. The post hoc analysis of the main effect CL stage is illustrated in Figure 1.

As shown, luteal cells from the early CL stage produced more P_4 than those from the mid and late CL stages ($P<0.001$). Luteal cells from the mid CL stage produced more P_4 than those from the late CL stage on Days 3–5 of culture and in the sum of Days 0–3 plus Days 3–5 ($P<0.05$). Post hoc analysis of the main effect culture medium is illustrated in Figure 2. As shown, luteal cells (all stages pooled) cultured in TCM produced more P_4 than those cultured in SOF on Days 3–5 of culture ($P<0.05$). The effect of medium refreshment and the interactions refreshment by CL stage and refreshment by culture medium were not significant. These two latter effects are also illustrated in Figures 1 and 2, respectively.

Figure 1: Effect of CL stage (early, mid, and late) on P_4 production by luteal cells in culture under oil overlaying. To illustrate this effect, data of the culture media were pooled as the interaction CL stage by culture medium of the factorial analysis was non-significant. Bars represente the LSD means and the error bars the standard error of the mean. *0–3* Accumulated production of Days 0–3. *3–5* Accumulated production of Days 3–5 following medium refreshment on Day 3. Effect of medium Refreshment=*Σ0–5*−sum 0–3 plus 3–5. *5* Accumulated production of Days 0–5 without refreshment. ^{abc}P<0.05; *P<0.1 (tendency).

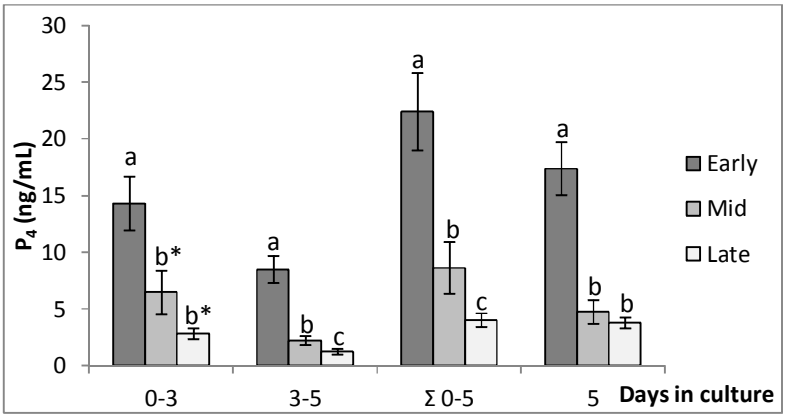
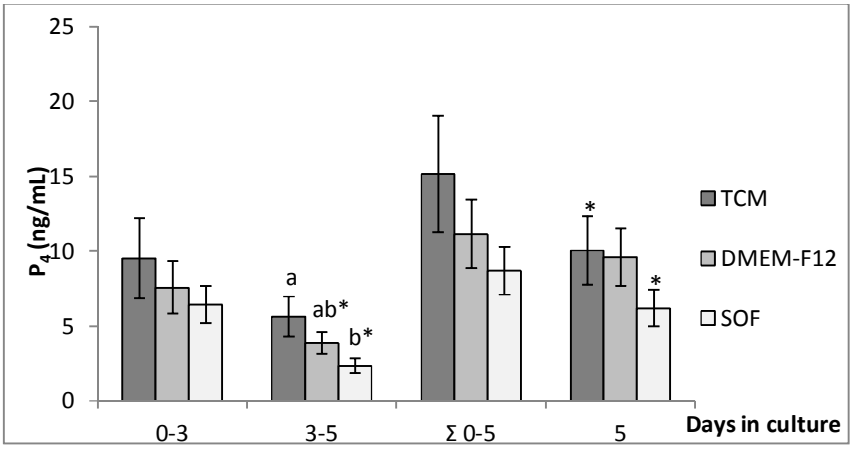


Figure 2: Effect of culture medium (TCM, DMEM-F12, SOF) on P_4 production by luteal cells in culture under oil overlaying. To illustrate this effect, data of CL stage were pooled as the interaction CL stage by culture medium of the factorial analysis was non-significant. Bars represent the LSD means and the error bars the standard error of the mean. *0–3* Accumulated production of Days 0–3. *3–5* Accumulated production of Days 3–5 following medium refreshment on Day 3. Effect of medium refreshment=*Σ0–5*−sum 0–3 plus 3–5. *5* Accumulated production of Days 0–5 without refreshment. ^{ab}P<0.05; *P<0.1 (tendency).



4.2 – Experiment 2

The main effect CL stage was significant, while the other main effects (culture medium, serum concentration, oxygen tension, and all interactions) were non-significant. As all interactions were non-significant, the data were pooled to illustrate each main effect. Figure 3 illustrates the main effect CL stage. As shown, early CL stage luteal cells had a significantly higher P_4 production than mid CL stage luteal cells ($P<0.00001$).

Figure 3: Effect of CL stage (early, mid) on P_4 production by luteal cells in culture without oil overlaying. To illustrate this effect, data of the culture media, serum concentration, and oxygen tension were pooled as all the interactions of the factorial analysis were nonsignificant. The concentrations of P_4 represent the daily production (i.e., Day 1 represents the accumulated production of Days 0–1 and so on). Refreshment of the culture medium was done daily after medium sample collection. Bars represent the LSD means and the error bars the standard error of the mean. $^{ab}P<0.00001$.

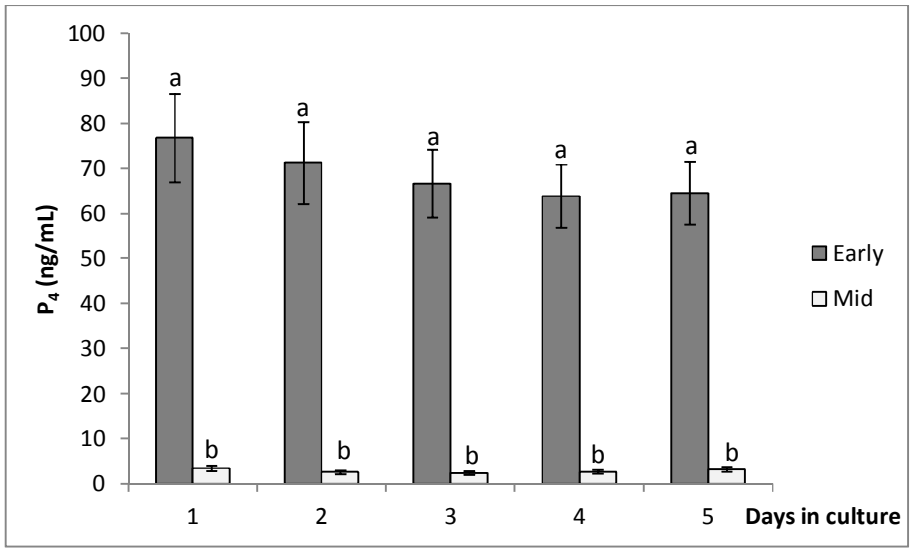


Figure 4A–C illustrates the effects of the culture medium, serum concentration, and oxygen tension, respectively. The factorial analysis evaluating the effect of culture medium refreshment showed that the effects of CL stage and the interaction CL stage by medium refreshment were significant.

Figure 4: Effects of medium (A), serum concentration in medium (B), and oxygen tension in culture atmosphere (C) on P_4 production by luteal cells in culture without oil overlaying. To illustrate these effects, data from the early and mid CL stages were pooled as all interactions of the factorial analysis were nonsignificant. The concentrations of P_4 represent the daily |production (i.e., Day 1 represents the accumulated production of Days 0–1 and so on). Refreshment of the culture medium was done daily after medium sample collection. Bars represent the LSD means and the error bars the standard error of the mean.

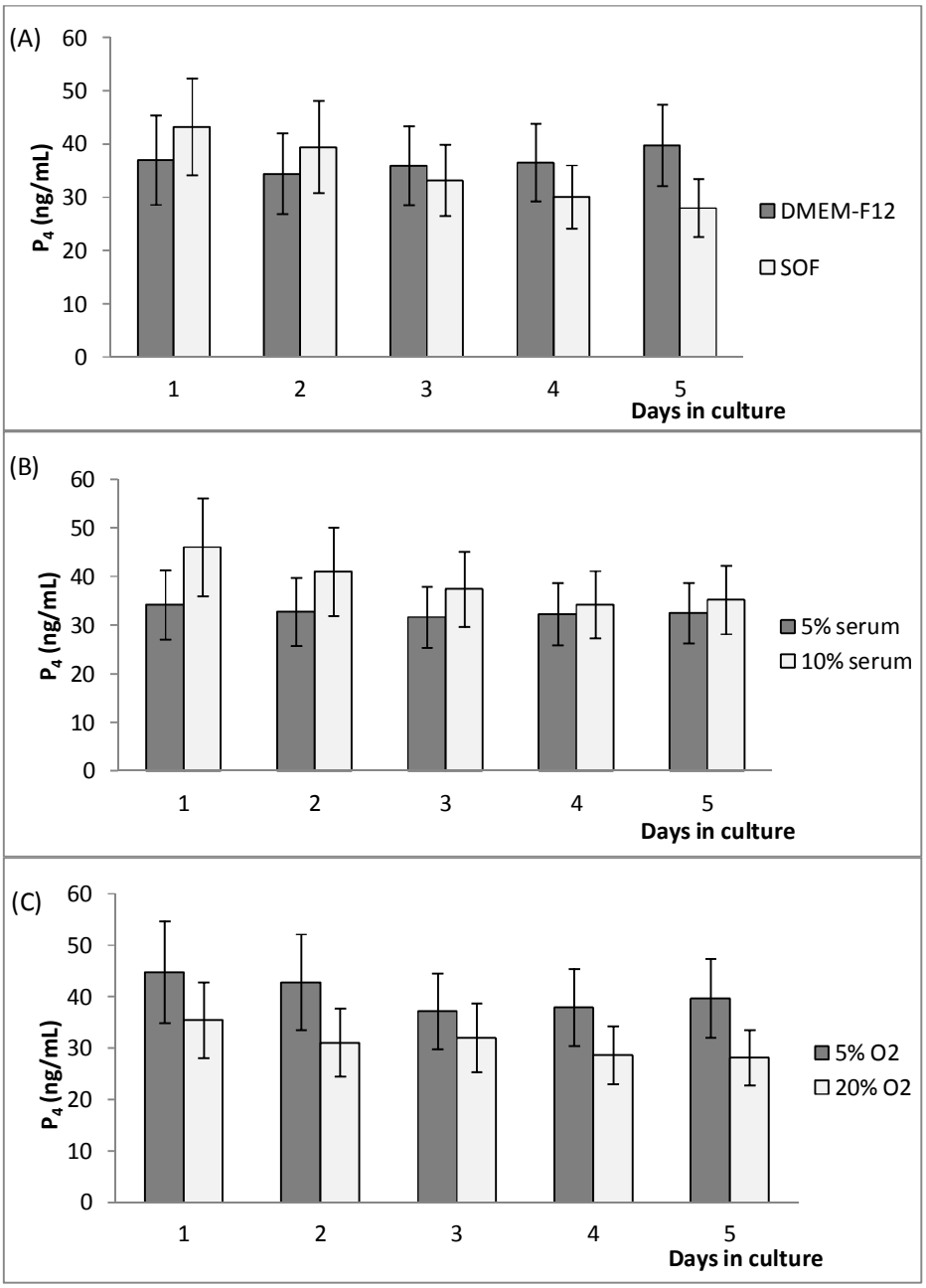
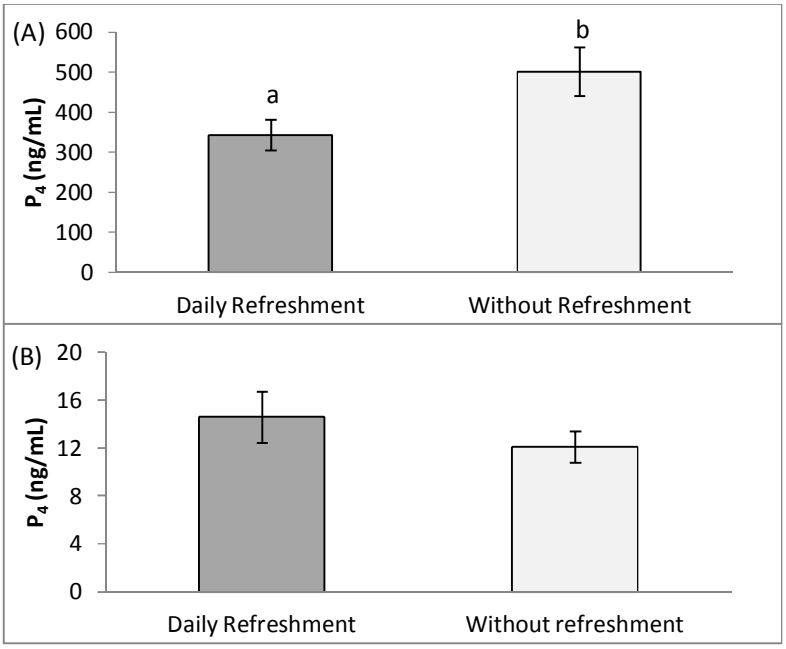


Figure 5 illustrates this latter interaction. As shown, daily refreshment of the culture medium negatively affected P_4 production of early CL stage luteal cells ($P<0.05$, panel a), but had no effect on mid CL stage luteal cells (panel b).

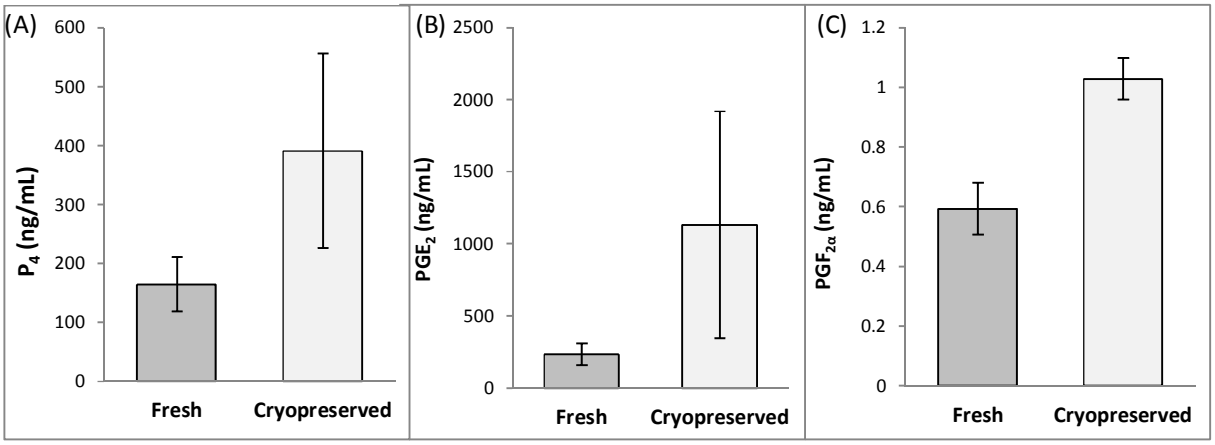
Figure 5: Effect of daily medium refreshment on the production of P4 by early (A) and mid (B) CL stage luteal cells in culture without oil overlaying. To illustrate this effect, data of the culture media, serum concentration, and oxygen tension were pooled as all the interactions of the factorial analysis were non-significant. Daily refreshment, Sum of P_4 produced at Days 1–5 (Day 1 represents the accumulated production of Days 0–1 and so on), refreshment of culture medium done daily after medium sample collection. Without refreshment, Accumulated P_4 production during 5 days of culture without medium refreshment. Bars represent the LSD means and the error bars the standard error of the mean. $^{ab}P<0.05$.



4.3 – Experiment 3

At the end point of culture (48 h), fresh and frozen–thawed cell cultures had similar mean cell numbers (6.6×10^4 versus 6.1×10^4cells/well, respectively, $P>0.05$). This represents a six-fold cell number increment per well. Also, the cell proliferation assay showed that the mean viability of fresh and frozen–thawed cell cultures was similar ($P>0.05$) at the end point of culture. As shown in Figure 6A–C, cryopreservation of early CL stage luteal cells had no significant effect on P_4, PGE_2, and $PGF_{2\alpha}$ production, respectively, after 48 h of culture.

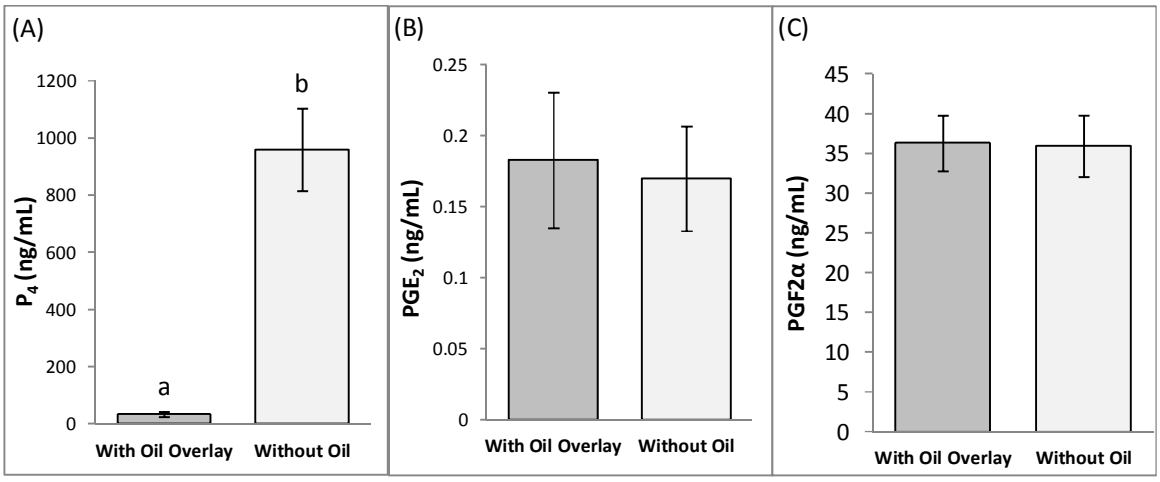
Figure 6: Effect of cryopreservation on P_4 (A), PGE_2 (B), and $PGF_{2\alpha}$ (C) production by early CL stage luteal cells in culture without oil overlaying. Bars represent the LSD means and the error bars the standard error of the mean.



4.4 – Experiment 4

As shown in Figure 7, the culture well oil overlay significantly ($P<0.00001$) affected the quantification of P_4 concentrations in the culture medium of cryopreserved early CL stage luteal cells (panel a), but had no effect on quantification of PGE_2 and $PGF_{2\alpha}$ concentrations (panels b and c, respectively).

Figure 7: Effect of oil overlay on the quantification of P_4 (A), PGE_2 (B), and $PGF_{2\alpha}$ (C) in the culture medium of cryopreserved early CL stage luteal cells. Bars represent the LSD means and the error bars the standard error of the mean. abP<0.05.



In experiment 3, significant correlations were observed between P_4 and $PGF_{2\alpha}$ (r=0.8, P=0.003), P_4 and PGE_2 (r=0.82, P=0.001), and $PGF_{2\alpha}$ and PGE_2 (r=0.74, P=0.006). In experiment 4, the correlation between the concentrations of $PGF_{2\alpha}$ and PGE_2 showed a tendency to significance (r=0.44, P=0.06).

5 – Discussion

In experiments 1 and 2, early CL stage luteal cells showed a significantly higher steroidogenic function than that of mid and late CL stage luteal cells. *In vivo*, early CL development following ovulation is characterized by the proliferation and differentiation of luteal cells in close contact with the capillary network (Reynolds & Redmer, 1999), and early CL stage luteal cells are those that putatively can interact with the early developing embryo. As these cells also showed the best adaptation to *in vitro* culture conditions, expressing the highest steroidogenic potential, they represent the best candidates to include in the luteal cell–embryo co-culture model.

Although refreshment of the culture medium once during the culture period had no effect on the steroidogenic function of cells (experiment 1), daily refreshment significantly decreased P_4 production of early but not mid stage CL luteal cells (experiment 2). Early CL stage luteal cells develop in hypoxic conditions, inside a less organized vascular network, their steroidogenic function being mainly dependent of their own production of stimulatory factors (Nishimura & Okuda, 2010). In contrast, mid CL stage luteal cells' steroidogenic function depends mainly on the endocrine and paracrine stimuli (Nishimura *et al.*, 2008). Daily refreshment of the medium in early CL stage luteal cell cultures might have removed autocrine factors, which jeopardized the steroidogenic function. Luteal cells were able to maintain the steroidogenic function in restrictive media used for early embryo culture. In fact, SOF was equally effective as DMEM-F12 in providing cell growth and function. Additionally, the steroidogenic function of early and mid CL stage luteal cells was not affected by a low content of serum in the culture medium, indicating that cholesterol availability (necessary for steroid production) is not a restrictive factor in a low-serum-content medium designed for embryo culture. These observations are relevant for the design of the co-culture model as complex media and high contents of serum were shown to induce epigenetic changes that led to abnormal embryonic and fetal development (reviewed by Farin *et al.*, 2006). Early and mid CL stage luteal cells were tolerant to low-oxygen tension atmospheres. This is not surprising for early CL stage luteal cells that develop in an *in vivo* hypoxic

environment. However, mid CL stage luteal cells develop in an *in vivo* rich oxygen environment provided by the mature well-vascularized CL. This result, indicating high *in vitro* developmental plasticity, partially conflicts with data reported by Nishimura & Okuda (2010) where hypoxic conditions (3% O_2) were detrimental to P_4 production by mid cycle luteal cells. Here (experiments 3 and 4), we report for the first time the use of previously frozen–thawed luteal cells in an *in vitro* experiment and their adequacy for the standardization of experimental conditions. Aliquots of frozen pools may be used in several culture sessions to avoid excessive variation in cell growing patterns and the function of primary cell cultures. The results indicated that cryopreservation did not affect cell viability and function (both steroidogenic and prostanoid).

Oil overlaying is routinely used in embryo culture to prevent evaporation and contamination. However, oil overlaying induced a 50-fold decrease in P_4 quantification in the culture medium (experiment 4). This was likely due to the lipophilic nature of this hormone allowing diffusion from the aqueous to the oil fraction, as previously reported (Shimada *et al.*, 2002; Clemente *et al.*, 2009). Therefore, oil overlay should be used with caution when studying steroidogenic interactions, especially if the expected steroid concentrations are low. Measurement of concentrations in the aqueous and oil fractions may be necessary. Interestingly, the quantification of prostaglandins was not affected by oil overlay. These compounds, in the past classified as lipophilic, did not behave as so, as also reported by Miller & Pursei (1987). Apart from that exposed above, experiment 4 considered all the *in vitro* culture conditions necessary for embryo culture and for studying the steroidogenic and prostanoid interactions in a co-culture model (medium, serum content, oxygen tension, incubation temperature, duration of culture, use of pools of cryopreserved early CL stage luteal cells, plasticware). These *in vitro* conditions sustained luteal cell growth, viability, and function.

In conclusion, the results reported here indicate that primary bovine luteal cells are able to support *in vitro* culture conditions suitable for early bovine embryo culture and maintain their steroidogenic and prostanoid functions. This will allow the use of a co-culture model to study the steroidogenic and prostanoid interactions between luteal cells and early embryos, which may be of relevance for the understanding of *in vivo* embryo development and pregnancy establishment in ruminant species.

Chapter II

Embryo – luteal cells co-culture: an *in vitro* model to evaluate steroidogenic and prostanoid bovine early embryo-maternal interactions

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1 – Abstract

The role of progesterone (P_4) and prostaglandins (PGs) in bovine early embryonic development and embryo-maternal cross talk is almost unknown. Here, the *in vitro* steroidogenic (P_4) and prostanoid (PGE_2 and $PGF_{2\alpha}$) interactions between bovine embryos and luteal cells (LC) were evaluated. In two experiments, embryos (n = 1.900) were either co-cultured with LC or cultured alone, from Day 2 to 7 (Day 0 = *in vitro* insemination). LC were also cultured alone and medium was used as a control, all groups being cultured either with or without oil overlay of culture medium. Oil overlay of culture medium significantly decreased the amount of P_4 but not of PGE_2 and $PGF_{2\alpha}$ measured in culture medium. Embryos and LC had transcripts of genes coding for enzymes of the PGs (PTGS2, PGES and PGFS) and P_4 (StAR, P450scc and 3β-HSD) synthesis pathways, and produced P_4, $PGF_{2\alpha}$ and PGE_2 into culture medium. Co-culture with LC exerted an embryotrophic effect, significantly increasing blastocyst yield and quality. This indicates a possible direct effect of LC in early embryo development. Embryos did not exert a luteotrophic effect upon LC. This may indicate that early embryos (until Day 7) probably do not exert influence in LC main function. It is suggested that production of P_4, PGE_2 and $PGF_{2\alpha}$ by early embryos may be associated to autocrine signaling leading to events in development and to paracrine signaling in the endometrium leading to local uterine receptivity.

Keywords: embryo; luteal cells; co-culture; progesterone; prostaglandins; bovine

2 - Introduction

In cattle, although embryo-maternal cross-talk associated to the onset of maternal recognition of pregnancy (around Day 16) is well characterized (Mann & Lamming, 2001; Mamo *et al*., 2012), early embryo-maternal crosstalk (until Day 8) leading to embryonic survival and pregnancy establishment is almost unknown (Forde *et al.,* 2011b). *In vivo* evaluation of this early embryo-maternal communication is difficult due to absence of systemic signals, extensive hormonal and biochemical background noise, and to animal welfare reasons. *In vitro* embryo-cells co-culture systems allow the evaluation of direct interactions (Pereira *et al.,* 2009; Orsi & Reischl, 2007), and an embryo-luteal cells (LC) co-culture model can potentially provide relevant data for the deciphering of early embryo-maternal crosstalk. A LC *in vitro* culture system suitable for embryo co-culture was previously developed (Batista *et al.,* 2012). The use of a long-term embryo-LC co-culture for evaluation of early embryo-luteal interactions has not been reported.

A putative direct interaction between the early embryo and LC may occur through the uterine-ovarian countercurrent transport mechanism (Einer-Jensen, 1988), which allows the transfer of prostaglandins (PGs), steroid hormones and peptides in many mammals (pig, sheep, cow and mice) (Einer-Jensen & Hunter, 2005). In ruminants, the uterine-ovarian countercurrent mechanism is pivotal for $PGF_{2\alpha}$ of endometrial origin reach high concentrations in the ovarian artery, thus triggering the luteolytic signal (Ginther, 1974). The ovarian-uterine countercurrent signal is critical for progesterone (P_4) of luteal origin to reach high concentrations in the uterine horn ipsilateral to the corpus luteum (Weems *et al.,* 1988). It is well established that more cows become pregnant when embryos are transferred to the anterior third of the uterine horn adjacent to the corpus luteum containing ovary than to other regions of the uterus (Sreenan, 1976). Therefore, we can hypothesize that mediators of luteal origin may reach the uterine lumen, and mediators of embryonic origin may diffuse through the endometrium, attain the submucosal vascular network and reach the corpus luteum. These hypothetical local embryo-LC crosstalk would probably not be detected at the peripheral circulation. Another hypothetical mechanism for early embryo-LC crosstalk would be indirect through changes at the endometrial level.

Prostaglandins (PGE_2, $PGF_{2\alpha}$, PGI_2) are produced by LC and post-hatching elongating bovine blastocysts. *In vitro* production of $PGF_{2\alpha}$ and PGE_2 from *in vivo* recovered bovine blastocysts was reported as early as Day 13 to 16 (Shemesh *et al*., 1979), Day 16 to 19 (Lewis, 1982), Day 12 to 15 (Hwang *et al*., 1988) and Day 10 (only PGE_2; Wilson *et al.*, 1992). Production of prostacyclin (PGI_2) was also reported as early as Day 15 (Hwang *et al.*, 1988) and Day 10

(Wilson *et al*., 1992). Also, transcripts for genes coding for enzymes of the PGs synthesis pathway were found in Day 7 *in vitro* produced bovine embryos (PTGS2, El-Sayed *et al.,* 2006; Clemente *et al.,* 2009; PTGS2 and PGES, Saint-Dizier *et al.,* 2011), and in hatched bovine blastocysts (PTGS2, Pereira *et al*., 2005a). PTGS2 was also immunolocalized in ovine expanded blastocysts' trophoectoderm cells (Charpigny *et al*., 1997). The role of early embryonic synthesized PGs on embryo development is poorly understood. In other species, early embryonic synthesis of PGs was associated to blastocyst hatching and implantation (Chida *et al.,* 1986; Sayre & Lewis, 1993; Kennedy *et al*., 2007).

Progesterone (P_4) produced by LC exerts an embryotrophic effect early in pregnancy, modulating the luteolytic signal (Mann & Lamming, 1995) and regulating conceptus growth, development (Garrett *et al.*, 1988) and synthesis of interferon tau (Mann & Lamming, 2001). This effect is modulated by the uterus through changes in the endometrial transcriptome and the composition of the histotroph (Clemente *et al*., 2009; Forde *et al.*, 2009). It is controversial whether P_4 also exerts a direct effect on the embryo. An embryotrophic effect was observed in some (mouse: Ghaemi *et al.*, 2008; bovine: Melro *et al.*, 2006; Ferguson *et al.*, 2012), but not all studies (bovine: Goff & Smith, 1998; Clemente *et al.*, 2009; Larson *et al.*, 2011), and a negative effect was even reported when using a BOEC co-culture system (Pereira *et al.,* 2009). Although *in vivo* recovered post-hatching elongating bovine blastocysts produced P_4 in *in vitro* culture (Shemesh *et al.*, 1979), it is unknown whether pre-eclosion embryos are able to produce P_4.

It is also unclear whether this pre-eclosion embryo is capable of enhancing luteal function by exerting a luteotrophic signal. Early bovine embryos enhanced P_4 production by *in vitro* co-cultured luteal explants (Thibodeaux *et al.*, 1994) and follicular granulosa cells (Vasques *et al.,* 1998; Pereira *et al.,* 2006). *In vivo*, significant differences in plasma P_4 concentrations between pregnant and non-pregnant females were observed as early as Days 5 to 7 of pregnancy (Mann & Lamming, 2001; Green *et al.*, 2005). However these early deviation in P_4 concentrations between pregnant and non-pregnant females might be attributed to luteal dysfunction rather than to presence of an early embryonic luteotrophic signal. In fact, a significant deviation in P_4 profiles between non-bred and pregnant (from artificial insemination and embryo transfer) virgin dairy heifers was only observed after Day 14, i.e. at the estimated onset of the luteolytic signal (Chagas e Silva & Lopes-da-Costa, 2005).

The objective of this study was to evaluate steroidogenic (P_4) and prostanoid ($PGF_{2\alpha}$ and PGE_2) interactions between bovine early embryos and LC, using *in vitro* co-culture models.

3 - Materials and methods

3.1 - Experimental design

In Experiment 1, Day 2 (Day 0 = in-vitro insemination) cleavage stage embryos (≥ 4 blastomeres; n = 900) were randomly allocated to one of four groups and *in vitro* cultured from Day 2 to Day 7 in 4-well dishes (ref. 144444; Nunclon, Nunc, Roskilde, Denmark; 25 embryos/group/well; 9 replicates): i) embryos (E); ii) embryos co-cultured with LC (E+LC); iii) embryos, oil overlay (E+O); iv) embryos co-cultured with LC, oil overlay (E+LC+O). Additionally, LC alone were cultured without and with oil overlay (groups LC and LC+O, respectively), and medium alone either without or with oil overlay was used as a control (groups M and M+O, respectively). Cumulus cells were carefully removed from embryos, to eliminate extra-embryonic sources of hormone production. At Day 7, embryos were evaluated for stage of development and morphological quality and medium samples were collected for measurement of P_4, PGE_2 and $PGF_{2\alpha}$ concentrations. Additionally, replicates of pools of 3 embryos at the blastocyst stage of development (group E: 5 replicates, 15 embryos; group E+LC: 5 replicates, 15 embryos) and replicates of LC (group LC: 5 replicates; group E+LC: 5 replicates) were used for RNA extraction and evaluation of transcription patterns of genes coding for enzymes of the PGs (PTGS2, PGES and PGFS) and P_4 (StAR, P450scc and 3β-HSD) synthesis pathways. Only embryos of quality grade I (excellent) and II (good) were allocated to RNA extraction.

In Experiment 2, Day 2 cleavage stage embryos (≥ 4 blastomeres; n = 1000) were randomly allocated to one of two groups and *in vitro* cultured from Day 2 to Day 7 in slide chambers without oil overlay (ref. 354108, BD Falcon™ Culture Slides, BD Biosciences Europe, Erembodegem, Belgium; 25 embryos/group/chamber; 20 replicates): i) embryos (E); ii) embryos co-cultured with LC (E+LC). Additionally, LC were also cultured alone (group LC) and medium alone was used as a control (group M). At Day 7, embryos were evaluated for stage of development, morphological quality and cell number, and medium samples were collected for P_4 measurement.

3.2 - Reagents and media

All reagents and media were supplied by Sigma-Aldrich Química, S.A. (Madrid, Spain), except otherwise stated.

3.2.1 – LC media preparation

Transport medium: Dulbeco's PBS (ref. 21300-017; Gibco, Life Technologies, Foster City, CA, USA), supplemented with 100 UI/mL penicillin plus 100 µg/mL streptomycin (ref. 15140-122; Gibco) and 1% w/v BSA (ref. A7906); washing medium: Hank's (ref. H4385) supplemented with 2.5 µg/mL amphotericin b (ref. A2942), 0.05 mg/mL gentamicin (ref. G1522); digestion medium: DMEM-F12 (ref 11039-021; Gibco) supplemented with 0.2% collagenase (ref. 10103578001; Roche Applied Science, Mannheim, Germany), 0.1% DNaseI (ref. D5025); culture medium: DMEM-F12 supplemented with 2.5 µg/mL amphotericin b, 0.05 mg/mL gentamicin, 0.1 % w/v BSA, 1 µL/mL ITS (ref. I3146), and fetal calf serum (FCS; ref. 26140-079, Gibco) added at a final 10 % (v/v) concentration; cryopreservation medium: DMEM-F12 supplemented with 10% v/v FCS, 10% v/v DMSO (ref. D2650); co-culture medium: identical to embryo culture medium.

3.2.2 – Media for *in vitro* embryo production

Transport medium: identical to LC transport medium; holding medium: TCM-199 (ref. M2154) supplemented with 25 mM HEPES (ref. H3784), 5 mM sodium bicarbonate (ref. S4019), 0.2 mM pyruvic acid (ref. P3662), 25 µg/mL amphotericin b, 5 USP/mL heparin (ref. H3393) and 1% v/v FCS; maturation medium: TCM-199 supplemented with 0.4 mM L-glutamine (ref. G5763), 0.05 mg/mL gentamycin, 1 µL/mL insulin-transferrin-sodium selenite, 10 UI/mL PMSG and 5 UI/mL hCG (PG600; Intervet International, Netherlands), and 15% v/v FCS; capacitation medium: modified Tyrode's medium (TALP) supplemented with 72.72 mM pyruvic acid and 0.05 mg/mL gentamycin; fertilization medium: TALP supplemented with 5.4 USP/mL heparin, 10 mM penicillamine (ref. P4875), 20 mM hypotaurine (ref. H1384), 0.25 mM epinephrine (ref. E1635) and gentamycin; culture medium: synthetic oviductal fluid (SOF) medium supplemented with aminoacids (BME, ref. B6766, 30 µL/mL and MEM, ref. M7145, 10 µL/mL), 0.34 mM tri-sodium-citrate (ref. 6448.1000, Merck Millipore, Darmstadt, Germany), 2.77 mM myo-inositol (ref. I7508), 1 µL/mL gentamycin, 1 µL/mL ITS and 5% v/v FCS (Holm *et al.,* 1999).

3.3 - LC preparation

Ovaries from Frisian crossbred heifers (age range: 12-16 months) with early corpora lutea (Day 2-6 of the estrous cycle; Ireland *et al.,* 1980; Miyamoto *et al.*, 2000) were collected post-mortem at the local abattoir and transported to the laboratory in transport medium cooled at 4ºC, within one hour. Procedures for LC isolation, culture, and cryopreservation were recently described (Batista *et al.*, 2012). Briefly, corpora lutea were washed, minced and digested for 60 min in digestion medium. Supernatant was removed and pellets centrifuged for 20 min at 400 × g in a discontinuous Percoll® (ref. P4937) gradient, designed to recover the steroidogenic cells enriched fractions (Fridén *et al*., 1999). Cells recovered from 18–27% and 27–45% interfaces were further submitted to sequential decreasing speed centrifugations, to discard fibroblasts and endothelial cells. The enriched LC suspension was evaluated for viability with the Trypan Blue exclusion method, seeded in culture medium and cultured in a flask until 90% of confluence. Cells were then trypsinized, re-suspended in cryopreservation medium, frozen and stored in liquid nitrogen until use. At Day 1 (Day 0 = *in vitro* insemination), cells were thawed, suspended in culture medium in 4-well dishes (2.5×10^4 cells/well; Experiment 1) or slide chambers (2.5×10^4 cells/chamber; Experiment 2) and incubated at 37ºC in a 5% CO_2 in humidified air atmosphere to form a monolayer. At Day 2 culture medium was replaced by co-culture medium and cells further incubated alone or co-cultured with embryos at 38.5ºC in a 5% CO_2, 5% O_2 and 90% N_2 humidified atmosphere for 5 days. Two pools of cryopreserved LC were used in the 2 experiments.

3.4 – *In vitro* embryo production

Ovaries were collected post-mortem at the local abattoir from Frisian crossbred heifers (age range: 12-16 months). Ovaries were transported to the laboratory in transport medium at 37ºC, within one hour. Follicles with 2 to 6 mm in diameter were aspirated and unexpanded cumulus–oocyte complexes (COCs) with at least three layers of compact cumulus cells and even cytoplasm were selected, washed in holding medium, placed in 400 µL of maturation medium in 4-well dishes (25 COCs/well) overlaid with 400 µL mineral oil (ref. M8410) and incubated at 39ºC in a 5% CO_2 in humidified air atmosphere for 24 hours. Following maturation, COCs were washed in fertilization medium and placed in 4-well dishes containing 400 µL of fertilization medium overlaid with 400 µL mineral oil and co-incubated with sperm at 39ºC in a 5% CO_2 in humidified air atmosphere for 48 hours. For *in vitro*

insemination, frozen-thawed semen from one bull with previously proven *in vitro* and *in vivo* fertility was used throughout the experiment. After thawing, semen was layered below capacitation medium in a test tube and incubated for one hour at 39ºC in a 5% CO_2 in humidified air atmosphere to allow recovery of motile sperm through the swim-up procedure (Parrish *et al*., 1995). After incubation, the upper two thirds of the capacitation medium were recovered, centrifuged at 200 × g for 10 minutes, the supernatant removed, and the sperm pellet re-suspended in fertilization medium for *in vitro* insemination. The sperm concentration per fertilization well was adjusted to1×10^6 sperm/mL and day of *in vitro* insemination was considered Day 0. On Day 2, cleavage stage embryos were denuded from remaining cumulus cells by vortexing and embryos with four or more blastomeres selected for *in vitro* culture. Embryos were washed in culture medium, randomly allocated to groups, and placed in culture wells (25 per well) containing 400 µL of culture medium, and incubated in a 5% CO_2 plus 5% O_2 plus 90% N_2 humidified atmosphere for 5 days.

3.5 - Embryo evaluation

On Day 7, embryos were evaluated for stage of development (CM: compact morula; YBL: young blastocyst; BL: blastocyst; EBL: expanded blastocyst) and morphological quality according to IETS guidelines (Stringfellow & Seidel, 1998). Rates of total blastocyst and of quality grade I+II blastocyst yield at Day 7 were calculated. In Experiment 2, embryo total cell number was determined. Briefly, embryos were first washed in PBS, the zona pelucida removed by a 2% pronase (ref. P8811) PBS solution, embryos further washed in PBS and incubated in culture medium supplemented with 20% FCS at 30 minutes at 39ºC in a 5% CO_2 in humidified air atmosphere. Embryos were then placed in PBS supplemented with 10 µl mL^{-1} Hoechst (bisBenzimide H 33258, ref. B2883) and 4% PFA (ref. P6148) at room temperature for 30 minutes. Finally, embryos were washed in PBS, embedded in a slide within a mowiol drop, gently flattened with a cover slip and observed through a fluorescence microscope.

3.6 - Measurement of P_4 concentrations

Medium samples were collected at Day 7 and stored at -20ºC until assay. The concentrations of P_4 were measured by a solid phase RIA assay using commercial kits (Coat-A-Count Progesterone kit, Siemens Healthcare Diagnostics, GmbH, Eschborn, Germany). The samples

were assayed in duplicate. Intra- and inter-assay coefficients of variation were calculated according to Rodbard *et al.* (1971). The inter-assay coefficients of variation were 14.2%, 8.9% and 4.7% for three different controls used in the assay (for concentrations of 1.9, 3.7, and 17.4 ng/mL, respectively; Multivalent Control Module, Siemens Healthcare Diagnostics). The intra-assay coefficient of variation was 7.1%.

3.7 - Measurement of PGs concentrations

Medium samples were collected at Day 7 into tubes containing a stabilizer (0.3 M EDTA (ref. E5134) plus 1% aspirin (ref. A2093), pH 7.4) and stored at -80ºC until assay. Medium PGE_2 concentrations were measured using a commercial enzyme immunoassay kit (PGE_2 Express EIA kit nº 500141, Cayman Chemical, Cayman Europe, Tallinn, Estonia). Medium PGF_{2a} concentrations were measured by an enzyme immuno-assay method described previously (Skarzynski *et al*., 1999). The intra-assay and inter-assay coefficients of variation were 14.3% and 2.5% for the PGE_2 assay and 12.5% and 7.0% for the $PGF_{2\alpha}$ assay, respectively.

3.8 - RNA extraction and Real Time PCR analysis

Embryonic RNA was extracted using the Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems, Life Technologies, Foster City, CA, USA), whereas LC RNA extraction was performed using the Rneasy Mini kit (Qiagen GmbH, Hilden, Germany). DNA digestion was performed with the RNase-free DNase Set (Promega, Wood Hollow road, Madison, USA). Concentration and purity of RNA were determined spectrophotometrically at 260nm and 280nm and RNA quality was assessed by visualization of 28S and 18S rRNA bands after electrophoresis through a 1.5% gel agarose with ethidium bromide staining. Samples were stored at -80°C until processing. Complementary DNA (cDNA) synthesis was obtained using the SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Life Technologies, Foster City, CA, USA), the reverse transcriptase (RT) reaction being performed in a total reaction volume of 20 µL, and the obtained RT products stored at -20ºC until Real-Time PCR amplification. Target genes included 3 genes coding for enzymes of the P_4 synthesis pathway (StAR protein, $P450_{scc}$, 3β-HSD) and 3 genes coding for enzymes of the PGs synthesis pathway (PTGS2, PGES, PGFS), using GAPDH transcription as an internal control. The primers were chosen using an online software package

(http://frodo.wi.mit.edu/primer3/input.htm) (Table 1). The housekeeping gene was chosen using the NormFinder software (Jensen & Ørntoft, 2004), after comparison of GADPH, β-actin and H2a.1. Real-Time PCR was performed in 96-well plates (ref. AB17500; Frilabo, Maia, Portugal) using the Power SYBR®Green Master Mix (ref. 4385612, Applied Biosystems), the StepOnePlus Real-Time PCR System (Applied Biosystems), and the universal temperature cycles: 10 min of preincubation at 95ºC, followed by 45 two-temperature cycles (15 s at 95ºC and 1 min at 60ºC). Melting curves were acquired (15 s at 95ºC, 30 s at 60ºC and 15 s at 95ºC) to ensure that a single product was amplified in the reaction. Data regarding relative mRNA quantification was analyzed with the real-time PCR Miner algorithm (Zhao & Fernald, 2005).

Table 1: Primer sequences, GenBank access number and size of PCR products of target genes analyzed by Real Time PCR.

Gene	**Primer sequences**	**GenBank** [acc. no.]	**PCR products** [bp]
PTGS2	5'TGGGTGTGAAAGGGAGGAAA'3 5'AAGTGCTGGGCAAAGAATGC'3	AF004944.1	127
PTGES	5'CCGAGGACGCTCAGAGACAT'3 5'AAAGCCCAGGAACAGGAAGG'3	NM_174443.2	122
PTGFS (20 alpha-hydroxysteroid dehydrogenase)	5'GGAGGACCCCAGGATCAAAG'3 5'CTCAGCAATGCGTTCAGGTG'3	S54973.1	130
StAR protein	5'GGTGGTGGCACGTTTTCAAT'3 5'CCTTGTCCGCATTCTCTTGG'3	Y17259.1	79
$P450_{scc}$	5'CAGCATATCGGTGACGTGGA'3 5'GGCCACCAGAACCATGAAAA'3	K02130.1	139
3β-HSD	5'TCCCGGATGAGCCTTCCTAT'3 5'ACTAGGTGGCGGTTGAAGCA'3	NM_174343.2	116
GAPDH (*Bos taurus*)	5'CACCCTCAAGATTGTCAGCA3' 5'GGTCATAAGTCCCTCCACGA3'	BC102589	103

3.9 – Statistical analysis

Data were analyzed through the statistical software Statistica7® (Statsoft, Tulsa, OK, USA, 2004). In both experiments, data on P_4, PGE_2 and $PGF_{2\alpha}$ concentrations were normalized by log-transformation and analyzed through general linear procedures, factorial ANOVA. In Experiment 1, analysis considered the fixed effects of embryo (presence versus absence), LC (presence versus absence), oil (presence versus absence) and their interactions (2 × 2 × 2 design), while in Experiment 2 analysis considered the fixed effects of embryo (presence versus absence) and LC (presence versus absence) (2 × 2 design). In Experiment 2, embryonic cell number was analyzed by factorial ANOVA, considering the fixed main effects of embryonic stage (CM versus YBL versus BL versus EBL), LC (presence versus absence), and their interactions (2 × 2 design). Post-hoc Fisher-LSD evaluations were computerized for all effects. Data on relative mRNA level of target genes (StAR, $P450_{scc}$, 3β-HSD, PTGS2, PGES, PGFS) were analyzed by ANOVA. Categorical data were analyzed by Fisher's exact test. Significance was tested at the 5% level ($P< 0.05$). Values are Mean ± SEM, unless otherwise specified.

4 – Results

Tables 2 and 3 present Day 7 total embryo production and quality, and kinetics of development, respectively of Experiment 1 (considering the 4-well dish *in vitro* culture system and also evaluating the effect of oil overlay of culture medium) and Experiment 2 (considering the slide chamber *in vitro* culture system without oil overlay of culture medium). Figure 8 represents the comparison between embryos cultured alone and co-cultured with LC regarding mean cell number of embryonic developmental stages, in Experiment 2. Figure 9 represents the transcription levels of genes coding for enzymes of the P_4 and PGs synthesis pathways (StAR, $P450_{scc}$, 3β-HSD, PTGS2, PGES, PGFS), comparing embryos cultured alone versus embryos co-cultured with LC (Figure 9-A) and LC cultured alone versus LC co-cultured with embryos (Figure 9-B). Figures 10 and 11 illustrate the post-hoc analysis of the interactions embryos × LC × oil (Figure 10, Experiment 1) and embryos x LC (Figure 11, Experiment 2) regarding concentrations of P_4. These figures were decomposed in two graphs (A and B) due to the disparity between the magnitude of P_4 concentrations produced by embryos and LC, however the represented statistical analysis (superscripts) apply simultaneously to both graphs. Figures 12 and 13 illustrate the post-hoc analysis of the

interaction embryos × LC × oil (Experiment 1) regarding PGE_2 and $PGF_{2\alpha}$ concentrations, respectively.

Table 2 – Influence of luteal cell co-culture and culture medium mineral oil overlaying on embryo development and quality in a 4-well dish *in vitro* culture system (Experiment 1).
Groups: E + O = embryos, oil overlaying; E = embryos; E + LC + O = embryos co-cultured with luteal cells, oil overlaying; E + LC = embryos co-cultured with luteal cells
Stage of development: CM = compact morulae; YBL = young blastocyst; BL = blastocyst; EBL = expanding blastocyst
Fisher's exact test: within columns, values with different superscripts differ significantly (ab, $P < 0.001$; ac, bc, $P < 0.05$)

Group	**n**	**Day 7 Embryos n (%)**	**Quality I+II n (%)**	**Stage of development**	
				CM+ YBL n (%)	**BL + EBL n (%)**
E + O	225	60 (27)^{b}	48 (80)^{c}	21 (35)^{a}	39 (65)^{a}
E	225	30 (13)^{a}	18 (60)^{a}	14 (47)^{ab}	16 (53)^{ab}
E + LC + O	225	66 (29)^{b}	61 (92)^{b}	24 (36)^{a}	42 (64)^{a}
E + LC	225	64 (28)^{b}	65 (86)^{bc}	41 (64)^{b}	23 (36)^{b}

Table 3 – Influence of luteal cell (LC) co-culture on embryo development and quality in a slide-chamber *in vitro* culture system (without oil overlay) (Experiment 2).
Stage of development: CM = compact morulae; YBL = young blastocyst; BL = blastocyst; EBL = expanding blastocyst
Fisher's exact test: * $P < 0.05$; ** $P < 0.00001$

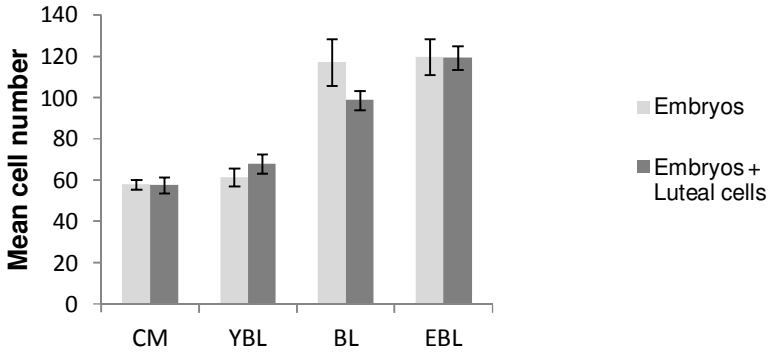
Group	**n**	**Day 7 Embryos n (%)**	**Quality I+II n (%)**	**Stage of development**	
				CM+ YBL n (%)	BL + EBL n (%)
Embryos	500	104 (21)*	62 (60)**	35 (38)	88 (64)
Embryos + LC	500	138 (28)*	122 (88)**	42 (36)	60 (58)

The factorial ANOVA models used in the statistical analysis showed that the effects of embryos, LC and oil (Experiment 1) and of embryos and LC (Experiment 2) on concentrations of P_4, PGE_2 and $PGF_{2\alpha}$ were significant. However, the interactions embryos x LC (Experiments 1 and 2) and embryos x LC x oil (Experiment 1) were not significant. Below follow the most relevant results.

4.1 - Luteal cells exerted an embryotrophic effect upon co-cultured embryos

These results were observed in both *in vitro* culture systems tested, the 4-well dish in Experiment 1 (Table 2) and the slide chamber in Experiment 2 (Table 3). Compared to embryos cultured alone (group E), co-culture with LC (group E+LC) increased Day 7 total embryo production (Experiment 1: $P< 0.001$; Experiment 2: $P< 0.05$) and embryo quality (Experiment 1: $P< 0.001$; Experiment 2: $P< 0.00001$), but had no effect on kinetics of development (Experiments 1 and 2) and embryo mean cell number (Experiment 2, Figure 8).

Figure 8: Effect of co-culture with luteal cells (LC) on Day 7 embryonic mean cell number of different stages of development. Error bars represent the standard error of the mean (SEM). Stages of development: CM = compact morulae; YBL = young blastocyst; BL = blastocyst; EBL = expanding blastocyst. No significant differences were observed between embryos cultured alone versus embryos co-cultured with LC ($P> 0.05$).



4.2 - Oil overlay of culture medium exerted an embryotrophic effect upon cultured embryos

In Experiment 1 (Table 2), in embryos cultured alone (group E), oil overlay of culture medium (group E+O) increased Day 7 total embryo production ($P< 0.001$) and embryo quality ($P< 0.05$), and significantly advanced embryonic development.

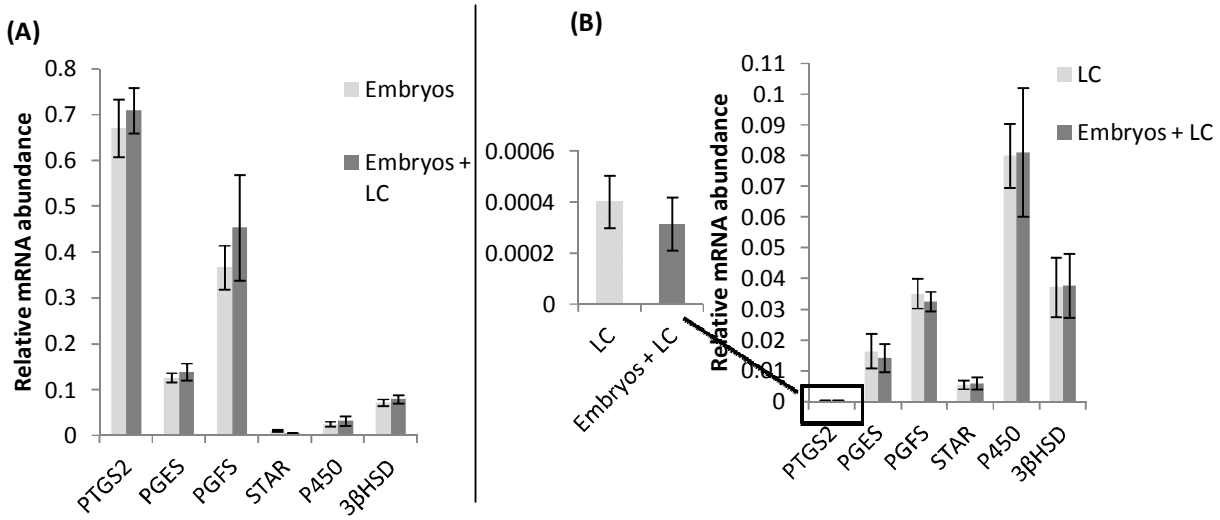
4.3 - The embryotrophic effects of LC and oil overlay of culture medium were not additive

In Experiment 1 (Table 2), Day 7 total embryo production and embryo quality were similar (P> 0.05) in embryos co-cultured with LC (group E+LC) and embryos co-cultured with LC and with oil overlay of culture medium (group E+LC+O).

4.4 - Pre-eclosion bovine embryos transcribed genes coding for enzymes of the P_4 synthesis pathway and produced P_4 into culture medium

In Experiment 1 (Figure 9-A), transcription of genes coding for enzymes of the P_4 synthesis pathway (StAR, $P450_{scc}$, 3β-HSD) was detected in embryos cultured alone (group E). In this experiment, as shown in Figure 10-A, compared to medium alone (group M), embryos cultured alone (group E) increased (P< 0.01) P_4 concentrations in culture medium. Also in Experiment 2 (Figure 11-A), compared to medium alone (group M), embryos cultured alone (group E) increased (P< 0.00001) P_4 concentrations in culture medium.

Figure 9: Illustration of the post-hoc LSD analysis of the interaction embryos × luteal cells (LC) on relative mRNA level for genes coding for enzymes of the prostaglandins (PTGS2, PGES, PGFS) and progesterone (StAR, $P450_{scc}$ and 3β-HSD) synthesis pathways, evaluated by Real-Time PCR. (A) - mRNA transcripts from embryos: comparison between embryos cultured alone versus embryos co-cultured with LC; (B) - mRNA transcripts from LC: comparison between LC cultured alone versus LC co-cultured with embryos. Error bars represent the standard error of the mean (SEM). No significant differences were observed between groups (P> 0.05).



4.5 - Pre-eclosion bovine embryos transcribed genes coding for enzymes of the PGs synthesis pathways and produced PGE_2 and $PGF_{2\alpha}$ into culture medium

In Experiment 1 (Figure 9-A), transcription of genes coding for enzymes of the PGs synthesis pathway (PTGS2, PGES, PGFS) was detected in embryos cultured alone (group E). In this experiment, compared to medium alone (group M), embryos cultured alone (group E) increased PGE_2 ($P< 0.01$; Figure 12) and $PGF_{2\alpha}$ ($P< 0.05$; Figure 13) concentrations in culture medium.

4.6 –Luteal cells transcribed genes coding for enzymes of the P_4 and PGs synthesis pathways and produced P_4, PGE_2 and $PGF_{2\alpha}$ into culture medium

In Experiment 1 (Figure 9-B), transcription of genes coding for enzymes of the P_4 and PGs synthesis pathways (StAR, $P450_{scc}$, 3β-HSD, PTGS2, PGES, PGFS) was detected in LC cultured alone (group LC). In this experiment, compared to medium alone (group M), LC cultured alone (group LC) increased P_4 ($P< 0.01$; Figure 10-B), PGE_2 ($P< 0.01$; Figure 12) and $PGF_{2\alpha}$ ($P< 0.05$; Figure 13) concentrations in culture medium. In Experiment 2 (Figure 11-B) compared to medium alone (group M), LC cultured alone (group LC) increased P_4 ($P< 0.00001$) concentrations in culture medium.

4.7 – Embryos of similar quality, either cultured alone or co-cultured with LC had similar transcription levels of genes coding for enzymes of the P_4 and PGs synthesis pathways

As shown in Figure 9-A, the transcription levels of genes coding for enzymes of the P_4 and PGs synthesis pathways (StAR, $P450_{scc}$, 3β-HSD, PTGS2, PGES, PGFS) were similar ($P> 0.05$) in embryos cultured alone (group E) and embryos co-cultured with LC (group E+LC).

Figure 10: Illustration of the post-hoc LSD analysis of the interaction embryos × luteal cells (LC) × oil overlaying of culture medium on progesterone (P_4) concentrations in Experiment 1. Figure was decomposed in two graphs due to the disparity of the magnitude of P_4 concentrations produced by embryos (A) and LC (B). Error bars represent the standard error of the mean (SEM). Columns with different superscripts differ significantly: abcd, $P < 0.01$. Mean values for groups LC+O and E+LC+O are presented within parenthesis.

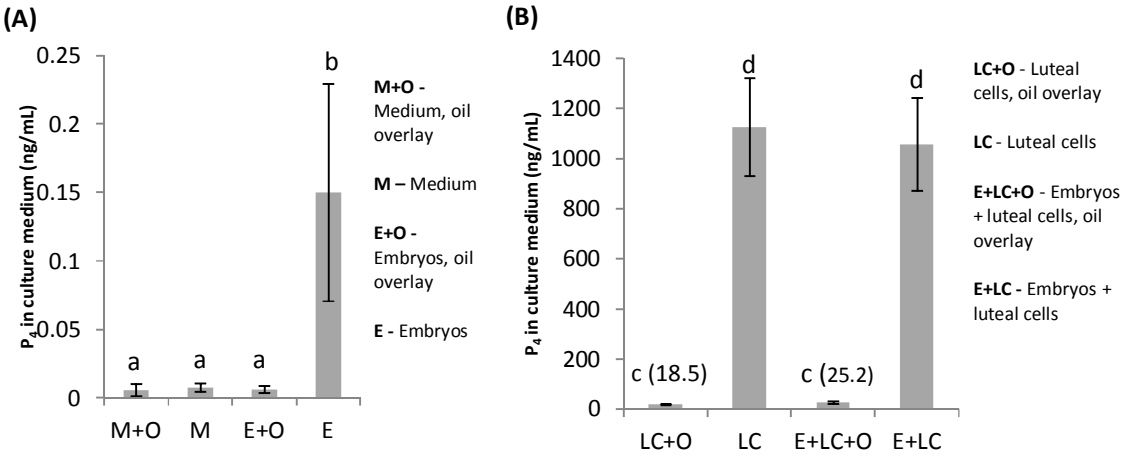


Figure 11: Illustration of the post-hoc LSD analysis of the interaction embryos × luteal cells (LC) on progesterone (P_4) concentrations in Experiment 2. Figure was decomposed in two graphs due to the disparity of the magnitude of P_4 concentrations produced by embryos (A) and LC (B). Columns with different superscripts differ significantly: abc, $P < 0.00001$

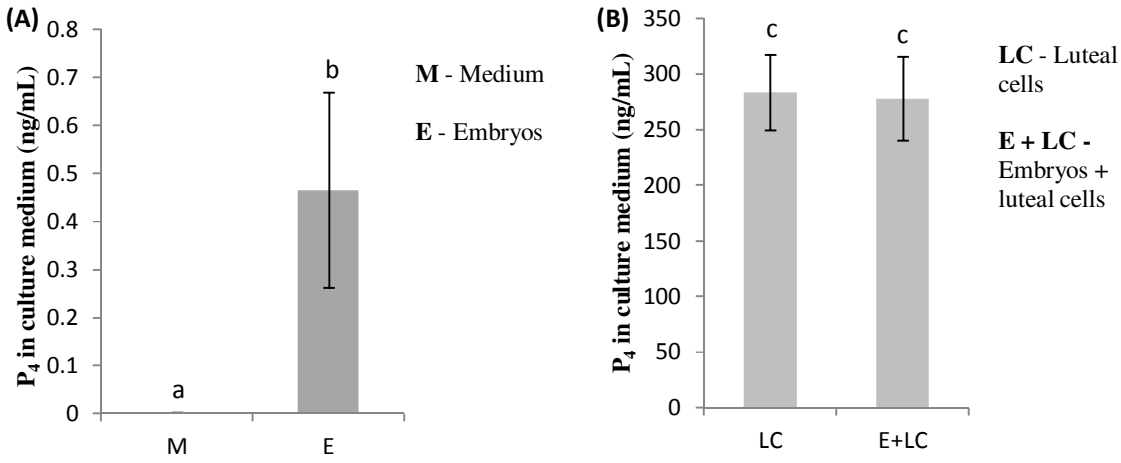


Figure 12: Illustration of the post-hoc LSD analysis of the interaction embryos × luteal cells (LC) × oil overlaying of culture medium on PGE_2 concentrations. Error bars represent the standard error of the mean (SEM). Columns with different superscripts differ significantly: ab, ac, cd, $P < 0.01$; ad, $P < 0.00001$; bc, bd, $P < 0.05$

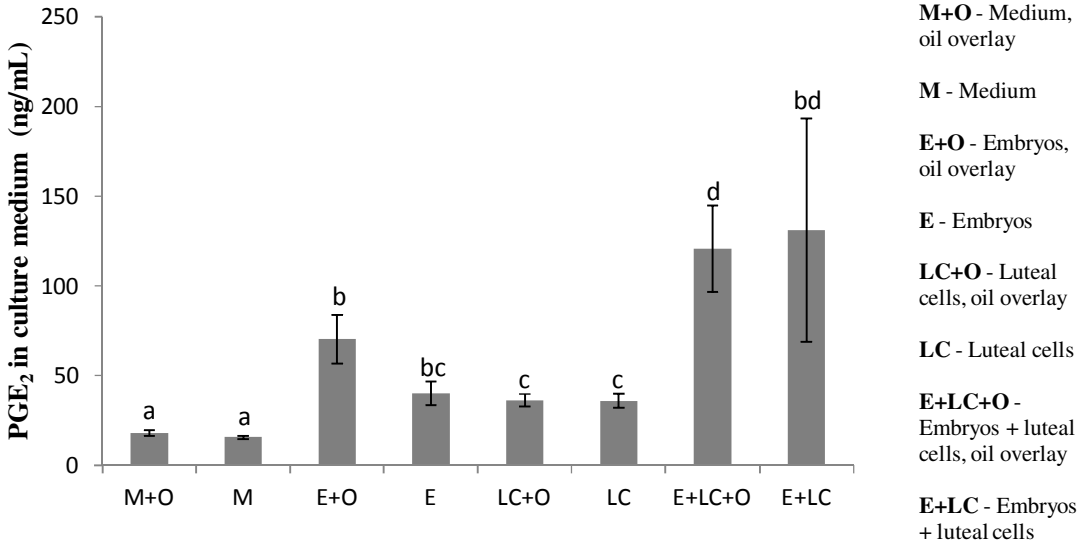
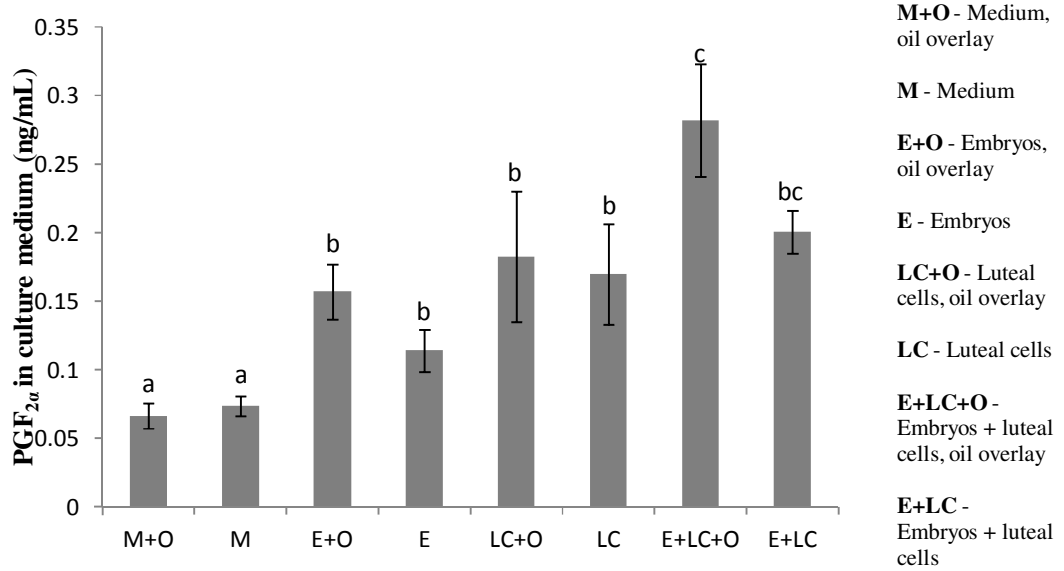


Figure 13: Illustration of the post-hoc LSD analysis of the interaction embryos × luteal cells (LC) × oil overlaying of culture medium on $PGF_{2\alpha}$ concentrations. Error bars represent the standard error of the mean (SEM). Columns with different superscript differ significantly: ab, $P < 0.05$; ac, $P < 0.00001$; bc, $P < 0.01$



4.8 – Luteal cells transcription levels of genes coding for enzymes of the P_4 and PGs synthesis pathways, and production of P_4, PGE_2 and $PGF_{2\alpha}$ into culture medium were not affected by co-culture with embryos

As shown in Figure 9-B, the transcription levels of genes coding for enzymes of the P_4 and PGs synthesis pathways (StAR, $P450_{scc}$, 3β-HSD, PTGS2, PGES, PGFS) were similar ($P> 0.05$) in LC cultured alone (group LC) and LC co-cultured with embryos (group E+LC).
Following the trend observed in gene transcription levels, LC cultured alone (groups LC and LC+O) and LC co-cultured with embryos (groups E+LC and E+LC+O, respectively) produced similar ($P> 0.05$) amounts of P_4 (Experiment 1: Figure 10-B; Experiment 2: Figure 11-B). Also, LC cultured alone (group LC) and LC co-cultured with embryos (group E+LC) produced similar ($P> 0.05$) amounts of $PGF_{2\alpha}$ (Figure 13).
Luteal cells cultured alone (groups LC and LC+O) produced less ($P< 0.01$) amounts of PGE_2 into culture medium than LC co-cultured with embryos (groups E+LC and E+LC+O) (Figure 12). However, as the interactions E x LC and E x LC x O of the statistical analysis were not significant, the higher amounts of PGE_2 produced by co-culture groups may represent an additive effect of the PGE_2 production of embryos and LC.

4.9 – Oil overlay of culture medium affected measurement of culture medium concentrations of P_4 but not of PGE_2 and $PGF_{2\alpha}$

As shown in Figure 10-AB, oil overlay of culture medium decreased ($P< 0.01$) the amount of P_4 measured in culture medium (comparisons of groups E versus E+O; LC versus LC+O; E+LC versus E+LC+O). As shown in Figures 12 and 13, oil overlay of culture medium had no effect ($P> 0.05$) on the amount of PGE_2 and $PGF_{2\alpha}$ measured in culture medium (through the same comparisons between groups).

6 – Discussion

6.1 – Embryonic development and quality

Co-culture with LC significantly increased blastocyst yield and quality. Somatic cells may stimulate embryonic development through the production of embryotrophic factors and/or

through the removal of toxic products of embryonic catabolism, protection against oxidative stress and modulation of medium physico-chemical properties (reviewed by Orsi & Reischl, 2007). This concept was explored in the early days of *in vitro* embryo production to by-pass the 4-cell stage blockage, due to inadequate cultural conditions at the time. However, the use of LC in embryo co-culture was not reported. Here, P_4 and PGs of LC origin might have exerted an embryotrophic stimulus, thus enhancing development. A direct positive effect of P_4 (Merlo *et al.*, 2006; Ferguson *et al.*, 2012) and PGE_2 (ovine: Sayre & Lewis, 1993; caprine: Sayre, 2007) on in *vitro* embryo development was reported. In the cow and ewe, early pregnancy P_4 supplementation enhanced embryonic growth, advanced development and increased *in vitro* secretion of interferon tau (Garrett *et al.*, 1988; Satterfield *et al.*, 2006; Carter *et al.*, 2008; Rizos *et al.*, 2012). In heifers, high circulating P_4 concentrations induced embryonic transcriptome changes, which could be associated to advanced post-hatching elongation (Carter *et al.*, 2010). However, in the above *in vivo* studies, a distinction between a direct effect of P_4 on the embryo and an indirect effect modulated by the uterus was not possible.

Oil overlaying of culture wells increased blastocyst yield and quality. Oil overlaying is a common procedure in in-vitro culture of embryos, protecting cultures from dehydration, pH variation, and accumulation of toxic components (Tae *et al.*, 2006). Interestingly, there was no additive embryotrophic effect following the use of co-culture and oil overlaying. This may indicate that the effects of these two components (LC and oil) were at some extent redundant. Therefore, the embryotrophic effect induced by co-culture with LC might have been due, partially, to a typical homeostatic somatic cell effect.

5.2 – Production of P_4 and PGs

Production of P_4 and transcription of genes coding for enzymes of the P_4 synthesis pathway were detected in bovine Day 7 embryos and LC. Production of P_4 by LC is well characterized in domestic mammals (Juengel & Niswender, 1999). Production of P_4 by the bovine conceptus between Days 13 to 16 of gestation (Shemesh *et al.*, 1979) and by pig (Stone *et al.*, 1986) and rabbit blastocysts (Dickmann *et al.*, 1975) was also reported. To the author's best knowledge, this is the first report to evidence P_4 production by Day 7 bovine blastocysts. As P_4 receptors (PR) are present in the bovine oviduct (Kenngott *et al.*, 2011), uterus (Robinson *et al.*, 2001; Okumu *et al.*, 2010), corpus luteum (Rueda *et al.*, 2000), and blastocyst (Clemente *et al.*, 2009), P_4 synthesized by the embryo may exert autocrine and/or paracrine

actions. Since the quantity of P_4 produced by a single Day 7 blastocyst is probably very low, it is plausible that P_4 of embryonic origin exerts an autocrine effect on development and a paracrine effect in the surrounding endometrium. This latter paracrine effect could be relevant for establishing an optimal uterine lumen microenvironment at the site of embryo entry into the uterus. Interestingly, LH receptors were detected in bovine oocytes and embryos, although their role in embryo development is unknown (Mishra *et al.*, 2003).

Production of PGE_2 and $PGF_{2\alpha}$ and transcription of genes coding for enzymes of the PGE_2 and $PGF_{2\alpha}$ synthesis pathways were detected in bovine Day 7 embryos and LC. These mediators are known to be *in vitro* produced at a 1:1 ratio by bovine LC from Day 15 of the estrous cycle onwards (Weems *et al.*, 1998), and by bovine post-hatching blastocysts from Day 12 onwards (Shemesh *et al.*, 1979; Hwang *et al.*, 1988). Recently, Saint-Dizier *et al.* (2011) reported the transcription of PGES in Day 7 bovine *in vitro* produced embryos, and a positive association of transcription levels with embryo quality. Here, to the best knowledge of authors, is reported for the first time the transcription of PGFS and the production of $PGF_{2\alpha}$ by pre-hatching bovine blastocysts. These mediators were suggested to be involved in blastocoel expansion and hatching in mouse (Baskar *et al*., 1981; Chida *et al.*, 1986; Huang *et al.,* 2004), ovine (Sayre & Lewis, 1993), goat (Sayre, 2007) and bovine blastocysts (Pereira *et al.,* 2005b). Receptors for PGE_2 and $PGF_{2\alpha}$ were identified in the bovine corpus luteum (Shariff *et al.*, 1998; Weems *et al.*, 2012), uterus (Arosh *et al.*, 2003, 2004b), and in the blastocyst ($PGF2_{2\alpha}$: Scenna *et al.*, 2006). Therefore, it is plausible that PGE_2 and $PGF_{2\alpha}$ exert an autocrine effect in blastocyst expansion and eclosion, and a paracrine effect in the uterus (and eventually in the corpus luteum, reaching the ovary through a countercurrent mechanism). PGs of luteal origin might also reach the uterine lumen through a countercurrent mechanism and exert a paracrine effect on the embryo.

Oil overlay of culture medium influenced embryonic production of PGE_2. Oil overlay numerically (although not significantly) increased PGE_2 production by embryos cultured alone, and significantly increased embryonic PGE_2 production compared to LC. This was probably associated to the embryotrophic effect exerted by oil, which significantly increased blastocyst formation, enhanced embryonic quality and advanced development. An increase in embryonic PGES transcription (and putatively in PGE_2 production) associated to an increase in embryonic quality was recently reported by others (Saint-Dizier *et al.*, 2011). A similar effect of oil overlay of culture medium on embryonic $PGF_{2\alpha}$ production cannot be depicted from results.

Embryos originated through culture alone and co-culture with LC had similar transcription levels of genes coding for enzymes of the P_4 and PGs synthesis pathways. This indicates that,

in good quality embryos reaching the end-point of in-vitro culture, co-culture with LC had no influence on embryonic transcription of the above genes. Gene transcription levels are probably related to embryonic quality, as reported for the PGES gene (Saint-Dizier *et al.*, 2011). Co-culture with LC induced a higher rate of blastocyst development and increased embryonic quality, compared to embryo culture alone. Therefore, it is plausible that a higher proportion of developing embryos with high transcription levels of target genes was present in the co-culture group than in the group of embryos cultured alone. This stimulation of embryonic gene transcription by LC co-culture could partially explain their embryotrophic effect. The effect of co-culture with LC on embryonic production of P_4, PGE_2 and $PGF_{2\alpha}$ is difficult to depict as the relative contributions of embryos and LC to steroid and prostanoid amounts in common culture medium are unknown. Nevertheless, the interactions embryos x LC were not significant, which might indicate that production of each co-culture member was not influenced by the other member's production. However, in the case of P_4 production, the proportion of the total amount produced by embryos in co-culture was probably very low. Therefore, small albeit significant increases in embryonic P_4 production, stimulated by LC, would not probably significantly affect overall P_4 production in co-culture. In the cases of PGE_2 and $PGF_{2\alpha}$, the significant increase in production observed in co-culture, compared to embryos and LC cultured alone, might reflect an additive effect.

Luteal cells cultured alone and co-cultured with embryos had similar transcription levels of genes coding for enzymes of the P_4 and PGs synthesis pathways. This indicates that transcription of these genes in LC was not stimulated by co-cultured embryos. The evaluation of the effect of co-culture with embryos on LC production of P_4, PGE_2 and $PGF_{2\alpha}$ presents the same interpretation problem above addressed for embryo production of those substances. However, at least in the case of P4 production, no luteotrophic effect of embryonic origin was detected. Thibodeaux *et al.* (1994) reported a significant increase in P_4 production by explants of luteal tissue from mid-cycle corpora lutea co-cultured with *in vivo* recovered cow blastocysts for 18 hours, compared to luteal explants alone. The inconsistency between the above results and those presented here may be associated with the duration of culture. A transient increase in P_4 production detected in a short-term (18 hours) culture may not be detected after a long duration culture (here, 5 days). Vasques *et al.* (1998), using a granulosa cells monolayer, detected a biphasic (at the 4-16 cell stage and at hatching) significant increment of P_4 production by these cells, when co-cultured with bovine *in vitro* produced embryos. In contrast, human zygotes co-cultured with autologous granulosa cells significantly inhibited production of estradiol and P_4 by these cells (Seifer *et al.*, 1996). Results from the present study indicate that no luteotrophic (progesteronic) effect of embryonic origin is

detected in LC until this early stage of development (Day 7). This corroborates results obtained in *in vivo* experiments comparing non-bred and pregnant (from artificial insemination and from embryo transfer) virgin dairy heifers (Chagas e Silva & Lopes-da-Costa, 2005).

Overlaying culture wells with mineral oil significantly decreased the amount of P_4 measured in culture medium, but had no effect on the amounts of PGE_2 and $PGF_{2\alpha}$ measured. Although P_4 was not measured in the oil fraction, this indicates that mineral oil might have absorbed lipid-soluble components of culture media such as P_4, as also suggested by others (Miller & Pursel, 1987; Batista *et al.*, 2012). This diffusion of lipophilic compounds towards the oil fraction can significantly affect their measurement in culture medium. This was particularly true for low amounts of P_4, such as those produced by embryos cultured under oil overlay.

In conclusion, bovine LC from early corpora lutea and Day 7 *in vitro* produced embryos have transcripts of genes coding for the enzymes of the P_4, PGE_2 and $PGF_{2\alpha}$ synthesis pathways, and produce these substances. An embryotrophic stimulus of LC origin was observed, which resulted in increased blastocyst yield and quality, indicating a possible direct effect of LC in early embryo development. In contrast, no luteotrophic effect of embryonic origin was detected. This may indicate that early embryos (until Day 7) probably do not exert influence in LC main function. It is suggested that production of P_4, PGE_2 and $PGF_{2\alpha}$ by early embryos may be associated to autocrine signaling leading to events in development and to paracrine signaling in the endometrium leading to local uterine receptivity. Oil overlay of culture medium affected measurement of P_4 but not of PGE_2 and $PGF_{2\alpha}$ concentrations in culture medium.

Chapter III

Effects of oocyte donor age and embryonic stage of development on transcription of genes coding for enzymes of the prostaglandins and progesterone synthesis pathways in bovine *in vitro*-produced embryos

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(Submitted to *Zygote*)

1 – Abstract

Prostaglandins (PGs) and progesterone (P_4) are involved in pregnancy establishment in cattle. However, the ability of early stage bovine embryos to produce PGs and P_4, and the role of these mediators in embryonic development and survival are unknown or poorly understood. Reproductive technologies allow the use of pre-pubertal (PP) oocyte donors for *in vitro* embryo production. However, the yield of viable blastocysts from PP calves is low, compared to that of post-pubertal cyclic (C) heifers. The objective of this study was to evaluate the transcription patterns of genes coding for enzymes of the PGs (PTGS2, PGES, PGFS) and P_4 (StAR, $P450_{scc}$, 3β-HSD) synthesis pathways in Day 7 *in vitro* produced embryos. The effects of oocyte donor age (PP versus C) and embryonic stage of development (compact morulae and young blastocysts, CM+YBL versus blastocysts and expanded blastocysts, BL+EBL) on target gene transcription levels were evaluated through Real Time PCR amplification in a 2 × 2 factorial design (20 replicates, n = 60 embryos).

Transcripts for PTGS2, PGES, PGFS and StAR were detected in all replicates, while transcripts of $P450_{scc}$ and 3β-HSD were not detected in 4 CM+YBL replicates. Transcript levels of StAR were not affected by heifer age and embryonic stage of development. Transcript levels of PTGS2, PGES, PGFS and $P450_{scc}$ were not affected by heifer age but were significantly higher in BL+EBL than in CM+YB. Transcript levels for 3β-HSD tended ($P = 0.06$) to be higher in C than in PP heifers, and were also significantly higher in BL+EBL than in CM+YB.

In conclusion, Day 7 bovine embryos show transcription of the genes coding for enzymes necessary to produce P_4, PGE_2 and $PGF_{2\alpha}$. The putative role of these mediators in blastocyst differentiation, expansion, hatching, and in early embryo-maternal crosstalk and embryonic survival is discussed.

Keywords: gene transcripts; prostaglandins; progesterone; embryo; bovine

2 – Introduction

Early embryonic mortality is a major source of economic loss in cattle. The highest prevalence of embryonic mortality occurs until Day 16 of pregnancy, probably earlier, until Day 8, in high-yielding lactating dairy cows (Diskin & Morris, 2008). Early embryonic survival is partly dependent on maternal uterine receptivity, which is primed by circulating/uterine progesterone (P_4) concentrations (Clemente *et al.,* 2009; Forde *et al.,* 2009). However, early embryonic survival is mainly dependent on the intrinsic developmental competence, which is associated to the proper activation of the embryonic genome and the ensuing transcription patterns (Kanka *et al*., 2012). The effects of early embryo-maternal crosstalk on maternal and embryonic changes leading to embryonic survival are poorly understood.

Age of oocyte/embryo donors affects embryonic developmental competence and survival. Pre-pubertal (PP) donors had a lower production rate of viable blastocysts, compared to that of adult donors (Khatir *et al*., 1996; Presicce *et al.*, 1997; Majerus *et al.*, 1999; Ptak *et al*., 1999). In sheep, embryos from PP donors showed a delay in development (Ptak *et al.*, 1999; Majerus *et al*., 2000; Leoni *et al.*, 2006a) and a low survival following embryo transfer (O'Brien *et al.,* 1997; Ptak *et al*., 1999; 2003; Kelly *et al*,. 2005). Additionally, changes in the expression of developmentally important genes were observed in embryos derived from PP cattle (Oropeza *et al*., 2004) and sheep (Leoni *et al*., 2006b).

Prostaglandins (PGs) are mediators known to be involved in several reproductive events. Prostaglandin synthase proteins (PTGS1 and PTGS2, also known respectively as COX-1 and COX-2) are rate-limiting enzymes that catalyze the conversion of arachidonic acid into PGH_2, the common precursor of all PGs. The downstream enzymes, PGE-synthase (PGES) and PGF-synthase (PGFS) catalyze the conversion of PGH_2 to PGE_2 and $PGF_{2\alpha}$, respectively (Helliwell *et al.*, 2004). In cattle, $PGF_{2\alpha}$ is the major luteolytic agent, whereas PGE_2 has luteoprotective and anti-luteolytic properties (Asselin *et al.*, 1996; McCracken *et al.*, 1999). PTGS2- and PGE receptor type 2 (EP2)-deficient mice show disturbances in ovulation, fertilization, embryonic development and implantation (Lim *et al.*, 1997; Matsumoto *et al.*, 2001).

Production of PGs by blastocysts cultured *in vitro* was reported in the bovine (Shemesh *et al.,* 1979), human (Holmes & Gordashko, 1980), rabbit (Dey *et al*., 1980), ovine (Hyland *et al.*, 1982), pig (Davis *et al.*, 1983), equine (Watson & Sertich, 1989; Weber *et al*., 1992) and mouse (Marshburn *et al.*, 1990) species. *In vitro* production of $PGF_{2\alpha}$ and PGE_2 from *in vivo* recovered bovine blastocysts was reported as early as Day 13 to 16 (Shemesh *et al.* 1979),

Day 16 to 19 (Lewis, 1982), Day 12 to 15 (Hwang *et al.,* 1988) and Day 10 (only PGE_2; Wilson *et al.,* 1992). Production of prostacyclin (PGI_2) was also reported as early as Day 15 (Hwang *et al.,* 1988) and Day 10 (Wilson *et al.,* 1992).

In ruminants, P_4 produced by the corpus luteum (CL) is necessary for the establishment and maintenance of pregnancy (Juengel & Niswender, 1999). Maternal post-ovulatory P_4 concentrations stimulate blastocyst growth and elongation and synthesis of interferon tau, thus inhibiting luteal regression and expulsion of the embryo from the uterus (Mann & Lamming, 2001). This effect of P_4 seems to be indirect, through changes in the endometrial transcriptome and composition of the histotroph (Lonergan, 2011b), although a direct effect on the embryo was also reported (Merlo *et al.,* 2006; Ferguson *et al.*, 2012). In luteal cells, cholesterol, the main substract for P_4 production, is transported from the cytoplasm into mitochondria through the steroidogenic acute regulatory protein (StAR), where the enzyme CYP11A1 (also known as $P450_{scc}$) conducts a side chain cleavage reaction to generate pregnenolone (P_5). Conversion of P_5 to P_4 is catalyzed by 3β-hydroxysteroid dehydrogenase (3β-HSD) at the endoplasmic reticulum (reviewed by Stocco *et al.*, 2007). *In vitro* production of P_4 by *in vivo* recovered Day 14 to 16 bovine blastocysts was reported (Shemesh *et al.*, 1979). Recently, we reported the production of $PGF_{2\alpha}$, PGE_2 and P_4 by *in vitro* bovine embryos cultured from Day 2 to 7 (Torres *et al.*, 2012).

The objective of this study was to evaluate the transcription patterns of genes coding for enzymes of the PGs (PTGS2, PGES, PGFS) and P_4 (StAR, $P450_{scc}$, 3β-HSD) synthesis pathways, in early (Day 7) *in vitro* produced bovine embryos. Additionally, the effects of oocyte donor age [PP versus post-pubertal cyclic (C)] and embryonic stage of development [compact morulae and young blastocysts (CM+YBL) versus blastocysts and expanded blastocysts (BL+EBL)] on target gene transcription levels were also evaluated.

3 - Materials and methods

3.1 - Reagents and media

All reagents and media were supplied by Sigma-Aldrich Química, S.A. (Madrid, Spain), except otherwise stated. Transport medium: Dulbeco's PBS (ref. 21300-017; Gibco, Life Technologies, Foster City, CA, USA), supplemented with 100 UI/mL penicillin plus 100 µg/mL streptomycin (ref. 15140-122; Gibco) and 1% w/v BSA (ref. A7906); holding medium: TCM-199 (ref. M2154) supplemented with 25 mM HEPES (ref. H3784), 5 mM

sodium bicarbonate (ref. S4019), 0.2 mM pyruvic acid (ref. P3662), 25 µg/mL amphotericin b (ref. A2942), 5 USP/mL heparin (ref. H3393) and 1% v/v fetal calf serum (FCS; ref. 26140-079; Gibco); maturation medium: TCM-199 supplemented with 0.4 mM L-glutamine (ref. G5763), 0.05 mg/mL gentamycin (ref. G1522), 1 µL/mL insulin-transferrin-sodium selenite (ITS; ref. I3146), 10 UI/mL PMSG and 5 UI/mL hCG (PG600; Intervet International, Netherlands), and 15% v/v FCS; capacitation medium: modified Tyrode's medium (TALP) supplemented with 72.72 mM pyruvic acid and 0.05 mg/mL gentamycin; fertilization medium: TALP supplemented with 5.4 USP/mL heparin, 10 mM penicillamine (ref. P4875), 20 mM hypotaurine (ref. H1384), 0.25 mM epinephrine (ref. E1635) and gentamycin; culture medium: synthetic oviductal fluid (SOF) medium supplemented with aminoacids (BME, ref. B6766, 30 µL/mL and MEM, ref. M7145, 10 µL/mL), 0.34 mM tri-Sodium-citrate (ref. 6448.1000, Merck,), 2.77 mM myo-inositol (ref. I7508), 1 µL/mL gentamycin, 1 µL/mL ITS and 5% v/v FCS (Holm *et al.*, 1999).

3.2 – *In vitro* embryo production

Ovaries were collected post-mortem at the local abattoir from Frisian crossbred PP (age range: 6-9 months) and C (age range: 16-18 months) heifers. Differentiation between PP and C status was based on age, size of the genital tract and presence of CLs. Ovaries were sent to the laboratory in transport medium at 37ºC, within one hour. Follicles with 2 to 6 mm in diameter were aspirated and unexpanded cumulus–oocyte complexes (COCs) with at least three layers of compact cumulus cells and even cytoplasm were selected, washed in holding medium, placed in 400 µL of maturation medium in 4-well dishes (ref. 7341175, Nunclon, Nunc, Roskilde,164 Denmark; 25 COCs/well) overlaid with 400 µL mineral oil (Sigma M-8410) and incubated at 39ºC in a 5% CO_2 in humidified air atmosphere for 24 hours. Following maturation, COCs were washed in fertilization medium and placed inside 4-well dishes containing 400 µL of fertilization medium overlaid with 400 µL mineral oil and co-incubated with sperm at 39ºC in a 5% CO_2 in humidified air atmosphere for 48 hours. For *in vitro* insemination, frozen-thawed semen from one bull with previously proven *in vitro* and *in vivo* fertility was used throughout the experiment. After thawing, semen was layered below capacitation medium in a test tube and incubated for one hour at 39ºC in a 5% CO_2 in humidified air atmosphere to allow recover of motile sperm through the swim-up procedure (Parrish *et al.*, 1995). After incubation, the upper 2/3 of the capacitation medium were recovered, centrifuged at 200 × g for 10 min, the supernatant removed and the sperm pellet re-

suspended in fertilization medium for *in vitro* insemination. The sperm concentration per fertilization well was adjusted to 1×10^6 sperm/mL and the day of *in vitro* insemination was considered Day 0. On Day 2, cleavage stage embryos were denuded from remaining cumulus cells by vortexing and embryos with 4 or more blastomeres selected for *in vitro* culture. These embryos were washed in culture medium, placed in 4-well dishes (25 per well) containing 400 µL of culture medium overlaid with 400 µL mineral oil, and incubated in a 5% CO_2 plus 5% O_2 in humidified air atmosphere for 5 days. On Day 7, embryos were evaluated for stage of development and morphological quality according to IETS guidelines (Stringfellow & Seidel, 1998). Only embryos classified as quality grade 1 (excellent) or grade 2 (good) were selected for RNA extraction.

3.3 – RNA extraction and Real Time PCR analysis

For RNA extraction and further analysis, embryos originating either from PP or C heifers were allocated to two developmental stage groups: CM+YBL and BL+EBL. Five replicates of pools of 3 embryos of each developmental stage and heifer groups (overall 20 replicates, n = 60 embryos) were used for RNA extraction using the Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems, Life Technologies, Foster City, CA, USA), samples being stored at -80°C until processing. DNA digestion was performed with the RNase-free DNase Set (Promega, Wood Hollow road, Madison, USA). Concentration and purity of RNA were determined spectrophotometrically at 260nm and 280nm and RNA quality was assessed by visualization of 28S and 18S rRNA bands after electrophoresis through a 1.5% gel agarose with ethidium bromide staining. Complementary DNA (cDNA) synthesis was obtained using the SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Life Technologies, Foster City, CA, USA), the reverse transcriptase (RT) reaction being performed in a total reaction volume of 20 µL, and the obtained RT products stored at -20ºC until Real-Time PCR amplification. Target genes included 3 genes coding for enzymes of the P_4 synthesis pathway (StAR protein, $P450_{scc}$, 3β-HSD) and 3 genes coding for enzymes of the PGs synthesis pathway (PTGS2, PGES, PGFS), and GAPDH as an internal control (housekeeping gene). The primers were chosen using an online software package (http://frodo.wi.mit.edu/primer3/input.htm; Table 4).

Table 4: Primer sequences, GenBank access number and size of PCR products of target genes analyzed by Real Time PCR.

Gene	Primer sequences	GenBank [acc. no.]	PCR products [bp]
PTGS2	5'TGGGTGTGAAAGGGAGGAAA'3 5'AAGTGCTGGGCAAAGAATGC'3	AF004944.1	127
PTGES	5'CCGAGGACGCTCAGAGACAT'3 5'AAAGCCCAGGAACAGGAAGG'3	NM_174443.2	122
PTGFS (20 alpha-hydroxysteroid dehydrogenase)	5'GGAGGACCCCAGGATCAAAG'3 5'CTCAGCAATGCGTTCAGGTG'3	S54973.1	130
StAR protein	5'GGTGGTGGCACGTTTTCAAT'3 5'CCTTGTCCGCATTCTCTTGG'3	Y17259.1	79
$P450_{scc}$	5'CAGCATATCGGTGACGTGGA'3 5'GGCCACCAGAACCATGAAAA'3	K02130.1	139
3β-HSD	5'TCCCGGATGAGCCTTCCTAT'3 5'ACTAGGTGGCGGTTGAAGCA'3	NM_174343.2	116
GAPDH (*Bos taurus*)	5'CACCCTCAAGATTGTCAGCA3' 5'GGTCATAAGTCCCTCCACGA3'	BC102589	103

The housekeeping gene was chosen using the NormFinder software (Jensen & Ørntoft, 2004), after comparison of GADPH, β-actin and H2A.1. Real-Time PCR was performed in 96-well plates (ref. AB17500; Frilabo, Maia, Portugal) using the Power SYBR®Green Master Mix (ref. 4385612, Applied Biosystems), the StepOnePlus™ Real-Time PCR System (Applied Biosystems), and the universal temperature cycles: 10 min of preincubation at 95ºC, followed by 45 two-temperature cycles (15 s at 95ºC and 1 min at 60ºC). Melting curves were acquired (15 s at 95ºC, 30 s at 60ºC and 15 s at 95ºC) to ensure that a single product was amplified in the reaction. Data regarding relative mRNA quantification was analyzed with the real-time PCR Miner algorithm (Zhao & Fernald, 2005).

3.4 – Statistical analysis

Data were analyzed through the statistical software Statistica7 (Statsoft, Tulsa, OK, USA, 2004) with the general linear models and analysis of variance (ANOVA) procedures. The experiment considered a 2 × 2 factorial design, including the main fixed effects of heifer age (PP versus C), embryonic stage of development (MC+YBL versus BL+EBL) and their interaction, on the relative mRNA level of target genes (StAR, $P450_{scc}$, 3β-HSD, PTGS2, PGES and PGFS). Significant effects were further analyzed post-hoc by the Fisher-LSD test. Significance was tested at the 5% level ($P< 0.05$).

4 – Results

For validation of Real Time PCR amplification, PCR products of all target genes were sequenced. For each of these genes sequencing revealed a 100% homology with the corresponding bovine sequence on the GenBank database. A single peak was obtained on the dissociation curves of each target gene.

Through qRT-PCR, transcripts of PTGS2, PGES, PGFS and StAR were detected in all replicates (n = 20; n = 60 embryos). Transcripts of $P450_{scc}$ and 3β-HSD were not detected in 2 replicates from PP heifers and in 2 replicates from C heifers, these 4 replicates corresponding to the CM+YBL stage of development.

Table 5 presents the results (P values) of the main effects of the factorial ANOVA model applied to the transcription levels of target genes, and Figures 14 and 15 illustrate the post-hoc analysis of these effects.

Table 5: Results (P values) of main effects of the factorial ANOVA model applied to the transcription levels of target genes.

	Main effect		
Target gene	Heifer age	Embryonic stage of development	Interaction age by stage
PTGS2	0.74	0.0001	0.64
PGES	0.82	0.01	0.63
PGFS	0.93	0.0001	0.93
StAR	0.39	0.24	0.92
$P450_{scc}$	0.66	0.0001	0.23
3β-HSD	0.06	0.00001	0.06

Figure 14: Post-hoc analysis of the relative mRNA levels of genes coding for enzymes of the steroidogenic synthesis pathway, of *in vitro* produced embryos of different developmental stages of either pre-pubertal or post-pubertal cyclic heifers. Target genes: StAR (a), $p450_{scc}$ (b) and 3β-HSD (c)
Groups (5 replicates, 3 embryos per replicate; total – 20 replicates, n = 60 embryos):
PP CM+YBL = pre-pubertal heifer, compact morulas plus young blastocysts
PP BL+EBL = pre-pubertal heifers, blastocysts plus expanded blastocysts
C CM+YBL = cyclic heifer, compact morulas plus young blastocysts
C BL+EBL = cyclic heifer, blastocysts plus expanded blastocysts
Column bars represent the LSD means and error bars represent the standard error of the mean (SEM).
Column bars with different superscripts differ significantly: ab, P< 0.01; cd, P< 0.003; ce, P< 0.00001; de, P< 0.02.

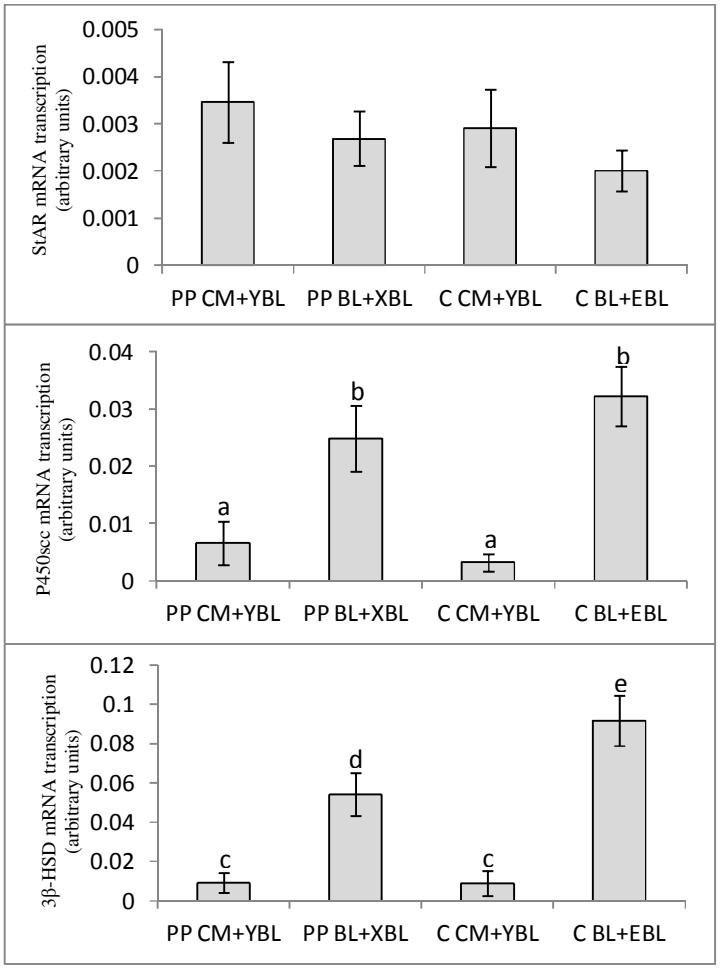
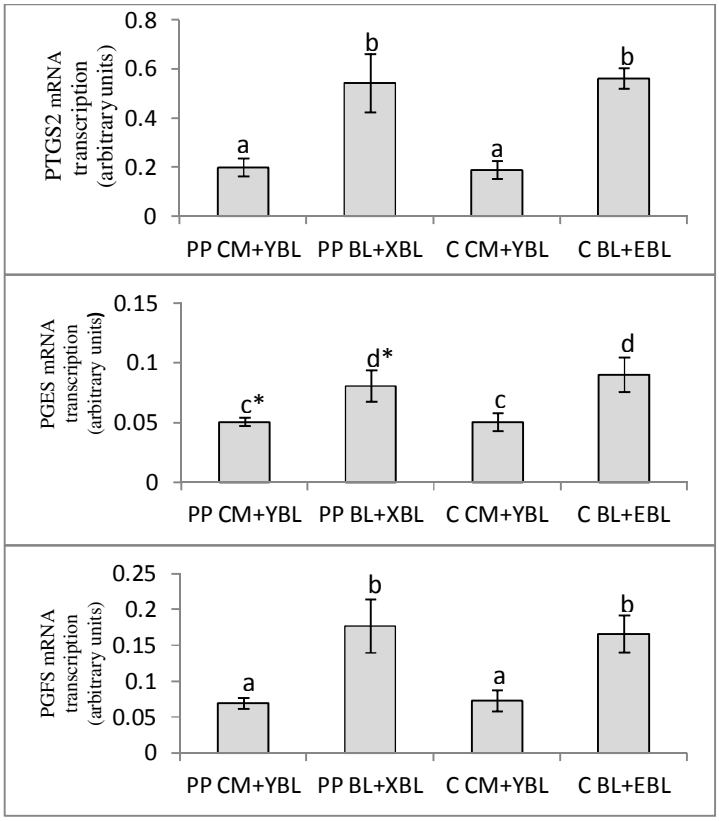


Figure 15: Post-hoc analysis of the relative mRNA levels of genes coding for enzymes of the prostaglandins synthesis pathway, of *in vitro* produced embryos of different developmental stages of either pre-pubertal or post-pubertal cyclic heifers. Target genes: PTGS2 (a), PGES (b) and PGFS (c)
Groups (5 replicates, 3 embryos per replicate; total – 20 replicates, n = 60 embryos):
PP CM+YBL = pre-pubertal heifer, compact morulas plus young blastocysts
PP BL+EBL = pre-pubertal heifers, blastocysts plus expanded blastocysts
C CM+YBL = cyclic heifer, compact morulas plus young blastocysts
C BL+EBL = cyclic heifer, blastocysts plus expanded blastocysts
Column bars represent the LSD means and error bars represent the standard error of the mean (SEM).
Columns with different superscripts differ significantly: ab, P< 0.01; cd, P< 0.05; * P= 0.06



Transcript levels of StAR were not affected by heifer age, embryonic stage of development and their interaction. Transcript levels of PTGS2, PGES, PGFS and $P450_{scc}$ were not affected by heifer age but were significantly affected by embryonic stage of development, being significantly higher in BL+EBL than in CM+YBL. Transcript levels of 3β-HSD were significantly affected by embryonic stage of development, being significantly higher in BL+EBL than in CM+YBL. Additionally, the main effects heifer age and the interaction heifer age × embryonic stage of development showed a statistical tendency (P = 0.06) to be significant, transcript levels of 3β-HSD being higher in C than in PP heifers.

5 – Discussion

Results here presented indicate that Day 7 bovine blastocysts already have transcripts for the enzymes necessary to produce P_4, PGE_2 and $PGF_{2\alpha}$. Transcription of PTGS2 was reported in *in vivo* Days 8 to 17 ovine embryos (Charpigny *et al.*, 1997), , *in vivo* Days 12 to 14 equine embryos (Aurich & Budik, 2005), *in vivo* murine morulae and blastocysts (Tan *et al*., 2005), *in vitro*-fertilized human morulae and blastocysts (Wang *et al*., 2002), and *in vitro* produced bovine Day 7 embryos (Clemente *et al.*, 2009; Saint-Dizier *et al*., 2011) and *in vitro*-fertilized bovine hatched blastocysts (Pereira *et al*., 2005a). In one report (Saint-Dizier *et al*., 2011), PTGS2 and PGES transcript levels were significantly lower in CM and YBL than in BL and EBL, which is in accordance with the results here presented. Increased transcription of PTGS2 and PGES at the first embryonic cell differentiation leads to the hypothesis that PGE_2 is involved in blastocoel formation and expansion, as suggested for the mouse model (Baskar *et al*., 1981; Huang *et al*., 2004). Also, the *in vitro* hatching rate of ovine embryos was increased in culture medium supplemented with PGE_2 (Sayre & Lewis, 1993), which prompts for a role of PGE_2 in blastocyst hatching.

Recently, we reported the production of $PGF_{2\alpha}$ by bovine embryos cultured *in vitro* from Days 2 to 7 (Torres *et al.,* 2012). Here is reported for the first time transcription of PGFS by Day 7 *in vitro* produced bovine embryos. The role of $PGF_{2\alpha}$ in embryonic development is controversial. Added to culture medium, $PGF_{2\alpha}$ decreased the rates of blastocyst yield and hatching in the rabbit (Maurer & Beier, 1976), rat (Buuck *et al*., 1997) and bovine (Fazio *et al*., 1997; Scenna *et al*., 2004) models. In contrast, Soto *et al.* (2003) reported no inhibitory effects of $PGF_{2\alpha}$ added to culture medium after fertilization on subsequent bovine embryonic development. A high ratio PGE_2: $PGF_{2\alpha}$ increased *in vitro* blastocyst hatching rates in the goat (Sayre, 2007).

In rodents, where implantation is invasive and occurs early after fertilization, PGs are major mediators in embryo implantation (Kennedy *et al.*, 2007). In ruminants, where implantation is non-invasive and gradual over weeks (reviewed by Weems *et al*., 2006), it is unlikely that PGs produced by early embryos act through the same mechanisms described in rodents. However, the bovine endometrium and myometrium synthesize receptors for PGE_2 and $PGF_{2\alpha}$ (Arosh *et al.*, 2003; 2004b). Therefore a paracrine effect of PGs of embryonic origin on the surrounding endometrium is plausible. This paracrine effect may induce changes in the local uterine lumen environment and composition of the histotroph that might be relevant to the establishment of uterine receptivity.

To the best knowledge of authors, here is reported for the first time the transcription of genes coding for enzymes of the P_4 synthesis pathway in *in vitro* Day 7 bovine embryos. Transcripts of $P450_{scc}$ and 3β-HSD were absent in some replicates of CM+YBL and transcription levels were significantly lower at those stages than at the BL+EBL embryonic stages. This indicates that these two genes start transcription at the time when blastomeres differentiate into the inner cell mass (ICM) and trophoectoderm cells. As P_4 receptor (PR) mRNA was detected at the blastocyst but not at the morula stage (Clemente *et al*., 2009), an autocrine/paracrine role of P_4 in the onset of the first embryonic differentiation is suggested. Endometrial expression of PR is high in the early luteal phase (Robinson *et al.*, 2001; Okumu *et al.*, 2010), but later on, in the mid luteal phase, P_4 induces downregulation of PR in endometrial epithelial cells (Bazer *et al.*, 2008). Therefore, it is plausible that P_4 of embryonic origin exert a paracrine effect in the surrounding endometrium, as discussed above regarding PGs. Interestingly, the presence of LH receptors in bovine embryos was reported (Mishra *et al.,* 2003), although their specific role in embryonic development is unknown.

Embryos originated from oocytes of PP and C heifers had similar transcription patterns of genes coding for enzymes of the PGs and P_4 synthesis pathways (except 3β-HSD). The developmental competence of embryos of PP heifers was reported to be donor age dependent (Armstrong, 2001). Older PP donors (7–11 months) have oocytes with a higher developmental competence (Presicce *et al.,* 1997; Tervit *et al.,* 1997; Majerus *et al.,* 1999) and embryos that achieve a higher pregnancy rate (Yang *et al.,* 1997), compared with younger PP heifers. Camargo *et al.* (2005) suggested that full developmental competence of PP oocytes is achieved at the age of 7 to 8 months, e.g., close to onset of puberty in *Bos taurus* breeds. As PP oocyte donors used in this study were in the above age range, this may explain the absence of age effects on the transcription levels of target genes. However, 3β-HSD transcript levels tended to be higher in embryos from C than in those from PP heifers. If embryonic synthesis of P_4 is linked to first embryonic differentiation and early embryo-maternal crosstalk (see above), then a low P_4 synthetic ability in PP heifer derived embryos might be associated to the reported disturbances in developmental kinetics (Ptak *et al.*, 1999; Majerus *et al.*, 2000; Leoni *et al.*, 2006a) and low *in vivo* survival (O'Brien *et al.,* 1997; Ptak *et al.*, 1999; 2003; Kelly *et al.,* 2005) of PP derived embryos.

In conclusion, Day 7 bovine embryos present transcripts of genes coding for enzymes of the PGs and P_4 synthesis pathways. Transcript levels (except those of the StAR gene) were affected by embryonic stage of development, being significantly higher in BL+EBL than in CM+YBL stages, which is coincidental with the first cellular differentiation within the developing embryo. This prompts for an autocrine/paracrine role of PGs in blastocoel

formation and expansion and for a paracrine role of both PGs and P_4 in the early embryo-maternal crosstalk. Age of oocyte donors had no significant effect on transcription levels of genes coding for enzymes of the PGs and P_4 synthesis pathways (except those of the 3β-HSD gene), which may be related to the use of older PP oocyte donor heifers. Transcript levels for 3β-HSD tended to be higher in embryos from C than in those from PP heifers. This may indicate a lower P_4 production ability of embryos from PP heifers, compared to those of C heifers, which might be of relevance for embryonic differentiation and survival.

Chapter IV

Secondary corpora lutea induced by hCG treatment enhanced demi-embryo survival in lactating high-yielding dairy cows

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1 – Abstract

Using a novel *in vivo* model considering a low developmental competence embryo (demi-embryo) and a sub-normal fertility recipient (lactating high-yielding dairy cow), this experiment evaluated the effect of human chorionic gonadotrophin (hCG) treatment at embryo transfer (ET) on embryonic size at implantation, embryonic survival and recipient plasma progesterone (P_4) and bovine pregnancy-specific protein B (PSPB) concentrations until Day 63 of pregnancy. Embryos were bisected and each pair of demi embryos was bilaterally transferred to recipients (n = 61) on Day 7 of the estrous cycle. At ET recipients were randomly assigned to treatment with 1500 IU hCG or to untreated controls.

Higher ($P< 0.01$) pregnancy rates on Days 25, 42 and 63, and embryo survival rate on Day 63 were observed in hCG-treated cows with secondary CL than in hCG-treated cows without secondary CL and in untreated cows. Pregnancy rates and embryo survival rate were similar in hCG-treated cows without secondary CL and untreated cows. Embryonic size on Day 42 was not affected by treatment with hCG, presence of secondary CL and type of pregnancy (single versus twin). Presence of secondary CL increased ($P< 0.05$) plasma P_4 concentrations of pregnant cows on Days 14, 19 and 25 but not thereafter, and of non-pregnant cows on Days 14 to 21. Treatment with hCG and presence of secondary CL had no effect on plasma PSPB concentrations, which were higher ($P< 0.05$) in twin than in single pregnancies.

In conclusion, secondary CL induced by hCG treatment at ET significantly increased plasma P_4 concentrations, the survival rate of demi-embryos and the pregnancy rate of high-yielding lactating dairy cows. Embryos were rescued beyond maternal recognition of pregnancy, but later embryonic survival, growth until implantation and placental PSPB secretion until Day 63 of pregnancy were not affected by treatment or presence of secondary CL.

Keywords: hCG, demi-embryo, PSPB, progesterone, bovine

2 – Introduction

High-yielding lactating dairy cows experience a high rate of steroid clearance, which induces low luteal peripheral progesterone (P_4) concentrations (Wiltbank *et al.*, 2006). A delay in the rise of post-ovulatory P_4 concentrations and low early luteal P_4 concentrations are associated to decreased blastocyst elongation, increased early embryonic loss and low pregnancy rates (Stronge *et al.*, 2005; McNeill *et al.*, 2006; Carter *et al.*, 2008). Early embryonic loss accounts up to 40% of AI losses (Humblot, 2001) and constitutes a serious source of reproductive failure and financial loss in dairy herds (Lucy, 2001). Due to the unequivocal role of P_4 on pregnancy establishment and maintenance, one of the strategies to increase embryo survival has been the enhancement of luteal function through the treatment with hCG (human chorionic gonadotrophin) (reviewed by Stevenson *et al.*, 2007; De Rensis *et al.*, 2010; Lonergan, 2011; Wiltbank *et al.*, 2011). Results have been controversial. Early luteal (Days 5 to 7) hCG treatment induces the formation of secondary CL and increases the peripheral concentrations of P_4 but this has not always been translated into an increase in pregnancy rate. Inconsistencies between reports may be explained by the concept that not all females/herds will benefit from treatment. High-yielding lactating dairy cows are a potential target of hCG treatment and, an increase in pregnancy rate of AI cows was reported (Santos *et al.*, 2001; Wiltbank *et al.*, 2011).

Treatment efficacy may also be related to embryo viability and formation of secondary CL. In virgin dairy heifers with healthy uteri and normal P_4 profiles, hCG treatment had no effect on embryo survival in AI heifers but increased pregnancy rate by 15% in frozen-thawed ET heifers developing secondary CL (Chagas e Silva & Lopes-da-Costa, 2005). This indicated that the secondary CL and/or the associated increased plasma P_4 concentrations may have compensated the lower viability of cryopreserved embryos. Demi-embryos originated by bisection have a higher rate of early and late embryonic mortalities than whole-embryos (Chagas e Silva *et al.*, 2008) and may be a good model to study the effect of hCG treatment on the survival of low viability embryos. However, early pregnancy P_4 supplementation had no effect on demi-embryo survival (Lopes-da-Costa *et al.*, 2011). This raises the hypothesis that the presence of secondary CL may be relevant for embryo survival, especially in the case of embryos with decreased viability.

In the present experiment we tested the main hypothesis that early luteal hCG treatment increases the survival of low viability embryos and that this effect is mediated through both the formation of accessory CL and the increase in plasma P_4 concentrations. Additionally, we

evaluated the effect of treatment on embryonic growth until implantation and on luteal and placental function until Day 63 of pregnancy.

3 – Material and Methods

3.1 – Experimental design

This experiment used an in vivo model that combines a low developmental competence embryo (demi-embryo; Chagas e Silva & Lopes-da-Costa, 2005) and a low fertility recipient (lactating high-yielding dairy cow; Lucy, 2001; Rodriguez-Martinez *et al*., 2008). This challenging model represents an ideal target to allow the expression of the putative beneficial effects of hCG therapy on embryo survival. In order to remove other sources of variation (genetics, nutrition, housing, etc.), the field experiment took place in a single herd, considering cows of similar physiological and production status, handled as a single group. These cows were randomly allocated to the treatment and control groups. To evaluate both the joint and separate effects of secondary CL and plasma P_4 concentrations on embryo survival, the three types of cows originated by hCG treatment were compared: i) hCG-treated with secondary CL; ii) hCG-treated without secondary CL but with putatively high plasma P_4 concentrations due to primary CL stimulation and, iii) untreated cows, without secondary CL and primary CL stimulation. As high-yielding dairy cows are more prone to double ovulations (Wiltbank *et al*., 2006), which could originate a distortion on the evaluation of effects of secondary CL and plasma P_4 concentrations, only single ovulating cows were allocated to the experiment. Demi-embryo bilateral transfer was chosen because this could potentially originate meaningful data relating the side of secondary CL formation to the survival of embryos. To avoid a putative effect of donor and sire on subsequent embryonic survival in cow recipients, each pool of donor selected embryos was equally distributed in the hCG and control transfer groups, so that each donor originated equal number of sets of pairs of demi-embryos for transfer in the two groups.

3.2 – Animals and treatments

The field tasks of the experiment were conducted in a single commercial dairy herd with an average of 500 Holstein cows at milking and an average milk yield of 11.500 Kg/cow/305 days lactation. The herd was involved in a herd health and reproductive veterinary program.

Dairy cows (n = 61) were enrolled in the study at their first spontaneous postpartum estrus after the voluntary waiting period (60 days) and after being considered reproductively sound on a routine palpation per rectum examination performed at that time. Cows were treated with a $PGF_{2\alpha}$ analogue (Veteglan, Laboratories Calier, Barcelona, Spain; 150 µg i.m.) to induce estrus and allocated to ET on Day 7 of the estrous cycle (Day 0 = estrus) after confirmation of the presence of a single mature CL. These 61 cows were randomly assigned to one of two groups: Treatment group received an i.m. injection containing 1500 IU of hCG (Chorulon, Intervet, Boxmeer, The Netherlands; (n = 30)) just before ET; Untreated (Control) group received no treatment (n = 31).

3.3 – Embryo production and bisection

Embryos were recovered from Holstein donors from the same herd and, upon bisection, the obtained demi-embryos were freshly bilaterally transferred to the uterine horns of recipients, according to methods previously described (Chagas e Silva & Lopes-da-Costa, 2005; Chagas e Silva *et al*., 2008). Briefly, donors were treated with Folltropin-V (Vetrepharma, London, Ontario, Canada; 400 mg i.m. given in eight decreasing doses, every 12 h, starting 9-11 days after a reference estrus) to induce superovulation, estrus was induced with Cloprostenol (Veteglan, Laboratories Calier, Barcelona, Spain; 150 µg i.m. at the time of the seventh and eighth gonadotrophin injections), AI was performed twice in the ensuing estrus and embryo recovery took place on Day 7 of the estrous cycle by routine non-surgical methods. The recovered uterine flushing medium was immediately filtered and the identified embryos were placed into culture medium (Vigro Holding Plus, AB Technology, Pullman, WA, USA) inside a four-well round bottom dish, washed and evaluated. Only embryos of IETS (Stringfellow & Seidel, 1998) quality grade 1 were allocated to the experiment.

One embryo at a time was washed in splitting medium (Vigro Splitting Medium, AB Technology, Pullman, WA, USA), moved into a 50 µL microdrop of the same medium and bisected through one microblade. Bisection was accomplished so that the two halves were of similar size and, for blastocysts so that the inner cell mass and the trophoectoderm cells were equally distributed in the two demi embryos. Upon bisection, the two halves were transferred to culture medium for 5 to 10 min, washed, evaluated, loaded in French mini-straws, and immediately transferred to recipients.

3.4 – Blood collection and storage

Blood samples were collected from the caudal vein in all recipients on Days 0 (estrus), 7 (embryo transfer and hCG injection if any), and 14, 19, 21 and 25. Pregnant cows on Day 25 were further submitted to blood collection on Days 35, 42, 49, 56 and 63 of pregnancy. All blood collections were performed at 09.00 AM before feeding (except on Day 0). Blood was collected into 10 mL heparin containing syringes (NH_4-heparin, Monovette, Sarstedt) through a 18 G needle, immediately centrifuged at 400 × g for 20 minutes, and the supernatant plasma pipetted into 1.5 mL cryotubes. These tubes were transported on ice to the lab and stored at -20ºC (for later analyses of concentrations of P_4) or -80ºC (for later analyses of concentrations of PSPB) until assay.

3.5 – Plasma P_4 measurement

Measurements were performed on all plasma samples collected on all sampling days. Plasma P_4 concentration was measured by a validated solid-phase RIA assay without extraction, using commercial kits (Coat-a-Count, Siemens Healthcare Diagnostics, GmbH, Eschborn, Germany). The analytical sensitivity of the assay was 0.02 ng/mL. Each sample (n = 504) was assayed in duplicate. The inter-assay coefficient of variation was 6.6% and 6.0% for pools with concentrations of 1.0 and 5.0 ng/mL, respectively. The overall intra-assay coefficient of variation was 3.5%.

3.6 – Plasma PSPB measurement

Measurements were performed in plasma samples collected on Days 7, 21, 25, 35, 42, 49, 56 and 63 of all Day 25 pregnant cows (n = 33). Measurement on Day 7 (before an embryo was transferred to the uterus) was intended to evaluate the presence of residual concentrations from previous pregnancy. Concentrations of PSPB were measured by ELISA ((Kit Elisa Dosage de Protéine de Gestation, UNCEIA, Maisons-Alfort, France); Jeanguyot *et al*., 2004). Each sample was assayed in duplicate. The analytical sensitivity of the assay was 7.8 pg/100 µL. The inter-assay and intra-assay CVs were 8%.

3.7 – Pregnancy evaluation

Presence of secondary CL (number and location) was evaluated on Day 14 in all recipients and pregnancy was evaluated on Days 25, 42 and 63 by ultrasonography, using a 7.5 MHz rectal linear probe. On Day 25, cows were considered as pregnant when one/two embryonic vesicles were observed and retrospectively confirmed by the presence of luteal plasma P_4 concentrations until Day 25 (the lowest plasma P_4 concentration of pregnant cows, measured until Day 25 was 3.93 ng/mL). On Day 42 an embryo proper with heartbeat was identified and size of the embryo was measured. The measurements included the crown-rump length (CRL) and the longest embryonic width (taken at mid body, at the stomach level; herein referred as width) and were taken twice, the resulting mean being recorded as the eligible measurement. On Day 63, the presence of a fetus with evident fetal activity and heartbeat was recorded.

3.8 – Statistical analysis

Data were analyzed through statistical software (Statistica 7, Statsoft, Tulsa, OK, USA, 2004), using general linear models procedures, analysis of variance (MANOVA) with repeated measures. The evaluation of the fixed effects of hCG treatment, presence of secondary CL and pregnancy on plasma P_4 concentrations included six levels of repeated measures for all cows (Days 0, 7, 14, 19, 21 and 25) and 11 levels of repeated measures for Day 42 pregnant cows (Days 0, 7, 14, 19, 21, 25, 35, 42, 49, 56 and 63), the effect of cow being considered as random. The evaluation of the fixed effects of hCG treatment and presence of secondary CL on PSPB concentrations of Day 42 pregnant cows included six levels of repeated measures (on Days 25, 35, 42, 49, 56 and 63). A MANOVA approach also evaluated the fixed effects of hCG treatment and presence of secondary CL on embryonic measurements (CRL and width). Post-hoc LSD evaluations were computerized for all effects. Correlations between concentrations of P_4, PSPB and embryonic measurements were calculated through the regressions computerized by the MANOVA models. Categorical data were analyzed by chi-square tests. Significance was tested at the 5% level ($P< 0.05$). Values are Mean ± SEM, unless otherwise specified.

4 – Results

4.1 – Development of secondary CL and pregnancy establishment

Data regarding the effect of hCG treatment on pregnancy establishment on Days 25, 42 and 63, proportion of twin pregnancies and embryo survival rate on Day 63 are presented in Table 6.

Table 6: Effect of hCG treatment on Day 7 of the estrous cycle on pregnancy establishment following demi-embryo bilateral transfer to high-yielding lactating dairy cows.

* number twin pregnancies / number pregnancies (%)

** number live conceptuses / number demi-embryos transferred (%)

For columns: ab, P< 0.01; cd, P< 0.05; ef, P= 0.06 (tendency)

Group	Pregnant – n (%)			Twins *	Embryo survival rate**
	Day 25	Day 42	Day 63	Day 63	Day 63
Untreated (n = 31)	11 (36) [a]	8 (26) [c]	8 (26) [c]	3/8 (38)	11/62 (18) [e]
hCG- treated (n = 30)	22 (73) [b]	16 (53) [d]	15 (50) [d]	4/15 (25)	19/60 (32) [f]

As expected, untreated cows did not develop secondary CL (only single ovulating cows were allocated to the study). From the 30 hCG-treated cows 18 (60 %) developed secondary CL, either ipsilateral (n = 15) or contralateral (n = 3) to the primary CL. Data regarding the effect of secondary CL on pregnancy establishment on Days 25, 42 and 63, proportion of twin pregnancies and embryo survival rate on Day 63 are presented in Table 7.

Table 7: Effect of secondary CL induced by treatment with hCG on Day 7 of the estrous cycle on pregnancy establishment following demi-embryo bilateral transfer to high-yielding lactating dairy cows.

sec CL = secondary CL

* number twin pregnancies / number pregnancies (%)

** number live conceptuses / number demi-embryos transferred (%)

For columns: ab, P< 0.0001; cd, P< 0.001; ef, P< 0.01

Group	Pregnant – n (%)			Twins *	Embryo survival rate**
	Day 25	Day 42	Day 63	Day 63	Day 63
Untreated (n = 31)	11(36)[a]	8(26)[c]	8(26)[e]	3/8(38)	11/62(18)[e]
hCG-treated without sec CL (n = 12)	4(33)[a]	2(17)[c]	2(17)[e]	0	2/24(8)[e]
hCG-treated with sec CL (n = 18)	18(100)[b]	14(78)[d]	13(72)[f]	4/13(31)	17/36(47)[f]

From the 15 cows that developed secondary CL ipsilateral to the primary CL, 10 (67%) established a pregnancy on Day 63, including three (30%) twin pregnancies. The three cows that developed contralateral secondary CL established a pregnancy on Day 63 (100 %), including one (33%) twin pregnancy. In untreated cows with single pregnancies one conceptus developed contralateral to the CL.

Embryonic size (CRL and width) on Day 42 was not affected by treatment with hCG (P = 0.83), presence of secondary CL (P = 0.85) and type of pregnancy (single versus twin; P = 0.69). Overall, Day 42 embryos had a mean ± SD CRL of 21.5 ± 2.3 mm and a mean width of 11.1 ± 1.4 mm.

4.2 – Plasma P_4 concentrations

Plasma P_4 concentrations were affected by hCG treatment ($P< 0.01$), presence of secondary CL ($P< 0.05$) and, as expected, by presence of pregnancy ($P< 0.00001$) and stage of pregnancy ($P< 0.00001$). The interactions hCG treatment by pregnancy and hCG treatment by stage of pregnancy were also significant ($P< 0.05$). Mean plasma P_4 concentrations on Day 7 (at treatment) were similar in hCG-treated and untreated cows (overall, mean ± SD: 3.48 ± 1.13 ng/mL; range: 1.13 - 6.04 ng/mL). On Day 14 (7 days after hCG treatment), treated cows with secondary CL had higher ($P < 0.0001$) mean plasma P_4 concentrations than untreated cows (mean ± SEM: 8.61 ± 0.52 ng/mL versus 6.06 ± 0.31 ng/mL, respectively) and hCG-treated cows without secondary CL ((6.84 ± 0.79 ng/mL; $P = 0.06$ (tendency)). However, mean plasma P_4 concentrations were similar in untreated and hCG-treated cows without secondary CL.

Figure 16: Effect of secondary CL induced by treatment with hCG on Day 7 of the estrous cycle on plasma P_4 concentrations of demi-embryo pregnancies (error bars represent the 95% confidence intervals). * $P< 0.05$.

with = Day 42 pregnant hCG-treated cows with secondary CL (n = 14); without = Day 42 pregnant untreated and hCG-treated cows without secondary CL (pooled data; n = 10).

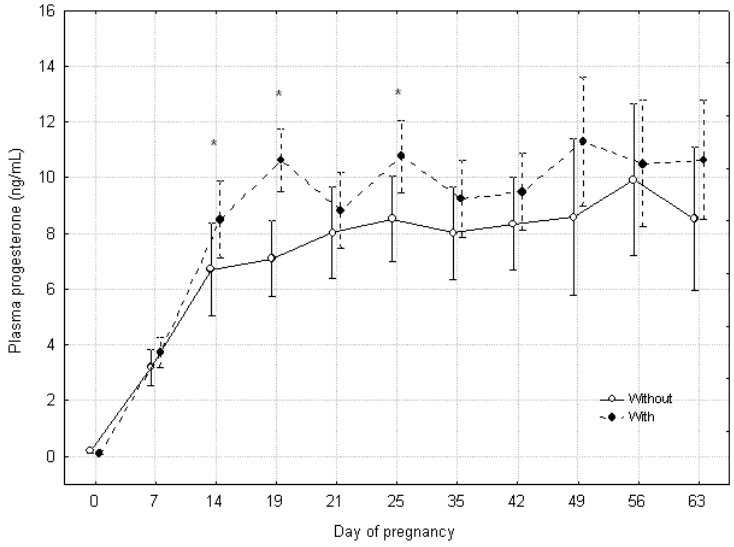
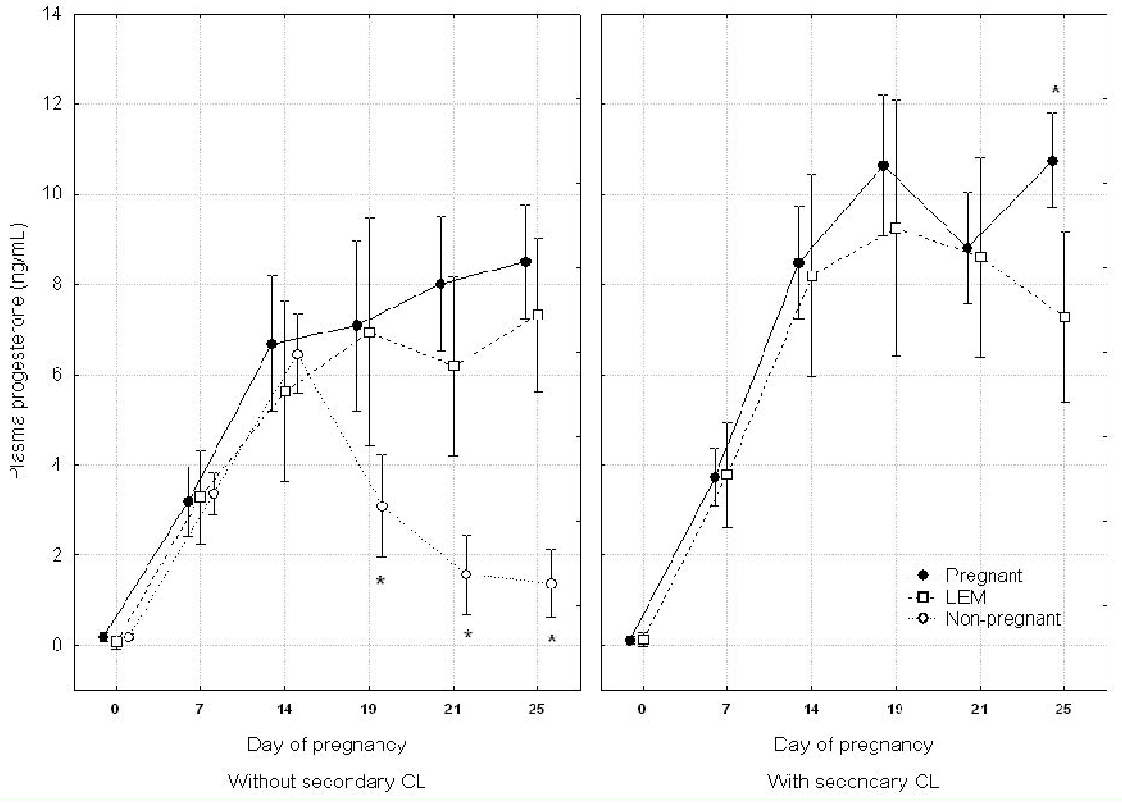


Figure 16 illustrates the effect of secondary CL on plasma P_4 concentrations of pregnant cows. As plasma P_4 concentrations of pregnant untreated cows and pregnant hCG-treated cows without secondary CL were similar, data were pooled (n = 10) to illustrate this effect.

As shown, pregnant cows with secondary CL had higher ($P < 0.05$) mean plasma P_4 concentrations than those without secondary CL on Days 14, 19 and 25 of pregnancy but not thereafter. Nine cows diagnosed pregnant on Day 25 were later found non-pregnant on Day 42 (four hCG-treated cows with secondary CL, two hCG-treated cows without secondary CL and three untreated cows, as can be depicted from Table 2) evidencing late embryonic mortality (LEM). Cows with LEM had plasma P_4 concentrations until Day 25 similar to those of cows remaining pregnant until Day 63. However, the Fisher-LSD post-hoc analysis showed that pregnant cows with secondary CL had higher ($P< 0.01$) plasma P_4 concentrations on Day 25 than LEM cows with secondary CL. These effects are illustrated in Figure 17. One cow pregnant on Day 42 was later found non-pregnant on Day 63, evidencing early fetal mortality. This cow had plasma P_4 concentrations typical of pregnant cows until Day 63.

Figure 17: Effects of late embryonic mortality (LEM) and secondary CL induced by treatment with hCG on Day 7 of the estrous cycle on plasma P_4 concentrations of demi-embryo pregnancies (error bars represent the 95% confidence intervals). * $P< 0.01$.



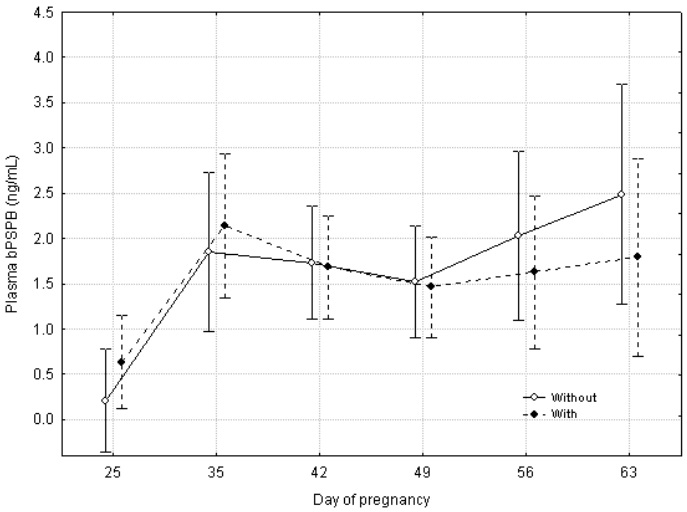
4.3 – Plasma PSPB concentrations

The nine cows diagnosed pregnant on Day 25 and later found non-pregnant on Day 42 were removed from subsequent analysis of pregnant cows to avoid a distortion of PSPB profiles of recipients remaining pregnant until Day 63. Plasma PSPB was detected in four of these cows and, in one of them up to Day 42. The cow pregnant on Day 42 and later found non-pregnant on Day 63 had detectable plasma PSPB concentrations until Day 49. Plasma PSPB was detected in five samples from Day 7. In cows remaining pregnant until Day 63 the PSPB detection rate was 9% (2/22 samples) on Day 21, 41% (9/22 samples) on Day 25 and 100% from Day 35 to Day 63.

Treatment with hCG and presence of secondary CL had no effect (P> 0.8) on plasma PSPB concentrations. Figure 18 illustrates the effect of secondary CL on plasma PSPB concentrations. As shown, plasma PSPB concentrations increased until Day 35, followed by a decrease until Day 49 and then raised again until Day 63.

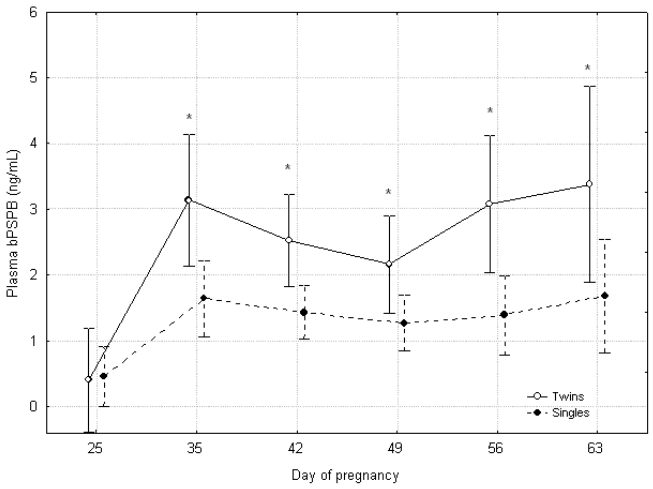
Figure 18: Effect of secondary CL induced by treatment with hCG on Day 7 of the estrous cycle on plasma PSPB concentrations of demi-embryo pregnancies (error bars represent the 95% confidence intervals).

with = Day 42 pregnant hCG-treated cows with secondary CL (n = 14); without = Day 42 pregnant untreated and hCG-treated cows without secondary CL (pooled data; n = 10)



Plasma PSPB concentrations from Day 35 to Day 63 were higher (P< 0.05) in twin than in single pregnancies (Figure 19).

Figure 19: Plasma PSPB concentrations of single (n = 16) and twin (n =7) demi-embryo pregnancies (error bars represent the 95% confidence intervals). * P< 0.05.



No significant correlation was found between P_4 and PSPB concentrations or between the concentrations of these hormones and embryonic measurements on Day 42.

5 – Discussion

5.1 – Pregnancy establishment

The demi-embryo survival rate here reported in untreated cows (18%) is similar to that obtained in beef cows with bilateral transfer (21%; *Davis et al.*, 1989) and indicates a low developmental competence of demi-embryos, as previously reported (Chagas e Silva *et al.*, 2008). Early luteal hCG treatment increased the proportion of Day 63 pregnant cows and Day 63 demi-embryo survival rate. These effects were already established at Day 25 of pregnancy, as no increase in the proportion of pregnant cows and in embryo survival rate were present beyond that stage of pregnancy. This indicates that hCG treatment was able to rescue embryonic development through the maternal recognition of pregnancy period, but had no effects thereafter. In beef cows, hCG treatment also increased pregnancy rate by 25% following the transfer of frozen-thawed embryos (Nishigai *et al.*, 2002) or by 8% with fresh and frozen-thawed embryos (Wallace *et al.*, 2011).

The high pregnancy rates achieved at Day 25 and Day 42 in hCG-treated cows may be related to the demi-embryo bilateral transfer method used in this experiment. Luteal stimulation through hCG treatment allowed for the early development of at least one of the demi-

embryos, which indicates that hCG treatment at ET is of merit to enhance the survival of low developmental competence embryos. However, contrary to expected, the proportion of twin pregnancies at Day 63 was similar in hCG-treated cows with secondary CL and untreated cows. This may indicate that LEM after Day 25 in hCG-treated cows mainly occurred in twin pregnancies.

The embryonic size attained by demi-embryos on Day 42 (at implantation) in lactating dairy cows is similar to that of both whole and demi-embryos previously reported in dairy heifer pregnancies (Lopes-da-Costa *et al.*, 2011). In the latter study (Lopes-da-Costa *et al.*, 2011) early luteal (Days 7 to 19) supplementation with P_4 significantly increased implantation embryonic size of demi-embryos (but not that of whole embryos). Results of the present study are different as hCG treatment, although significantly increasing plasma P_4 concentrations, had no effect on demi-embryo size at implantation. The previous route of P_4 administration (intravaginal) might have been instrumental for achieving the effect or, alternatively, the sample size here reported might not have allowed the expression of a statistically significant effect that was already subtle (5 to 10% increase in size). Implantation size of demi-embryos was similar in single and twin pregnancies. Fetal weight was similar in single and multiple pregnancies at Day 56 (Echternkamp, 1992) and at Day 95 (Bellows *et al.*, 1990) of pregnancy, but was higher in single than in multiple fetuses at Day 180 (Bellows *et al.*, 1990). Birth weight is usually significantly higher in single than in twin calves (Davis *et al.*, 1989; Echternkamp, 1992; Holland & Odde, 1992; Horta *et al.*, 1992; Sinclair *et al.*, 1995). Altogether, these data corroborate the concept that deviation in size between singletons and twins occur late in pregnancy, but the exact moment of the deviation is unknown.

5.2 – Development of secondary CL and plasma P_4 concentrations

The beneficial effect of hCG treatment on embryo survival was only present in cows developing secondary CL. This result suggests that formation of secondary CL is probably a critical requisite to induce a beneficial effect on embryo survival. This raises the hypothesis that secondary CLs secrete embryotrophic factors other than P_4, which may act locally on the uterus and/or embryo. However, the main effect of secondary CL was probably mediated by plasma P_4 concentrations. In hCG-treated cows with secondary CL, a significant increase in plasma P_4 concentrations was already noticeable on Day 14 and was extended until Day 25. This might have been critical to attenuate the luteolytic signal, thus extending the luteal phase and allowing extra time for the demi-embryo to elongate and secrete interferon tau. Early

luteal high peripheral P_4 concentrations attenuate the luteolytic signal (Mann *et al.*, 1998) and increase early embryonic growth, elongation and secretion of interferon tau (Garret *et al.*, 1988; Kerbler *et al.*, 1997; Mann & Lamming, 2001), which enhances early embryo survival. Results here presented support the concept that lactating high-yielding dairy cows benefit from luteal stimulation, as also reported by others (Santos *et al.*, 2001; Stevenson *et al.*, 2007; Wiltbank *et al.*, 2011). The role of P_4 on pregnancy establishment and maintenance is undisputed and was recently reviewed (Bazer *et al.*, 2010; Lonergan, 2011b). Progesterone is known to regulate endometrial gene expression and composition of the histotroph, thus indirectly affecting embryonic development (Clemente *et al.*, 2009; Forde *et al.*, 2009; Forde *et al.*, 2011a). Although hCG treatment at Day 5 would have primed the uterus with elevated P_4 concentrations at ET time, practical, logistical and animal welfare reasons make treatment at ET more convenient. In fact, when dealing with fresh transfers, in which estimation of donor viable embryo yield is unrewarding, anticipated treatment of surplus recipients can potentially occur. In hCG-treated cows with secondary CL the beneficial effect on embryo survival was established at Day 25 of pregnancy, and the significant increase in plasma P_4 concentrations occurred until Day 25. This indicates that P_4 from secondary CL is critical for embryo survival only until Day 25 of pregnancy and has minor if any effects in implantation rate and fetal survival. Cows experiencing LEM (from Day 25 until Day 42 of pregnancy) presented plasma P_4 concentrations until Day 25 similar to cows maintaining pregnancy until Day 63. Therefore, the cause of LEM cannot be attributed to an early deficiency in P_4 concentrations. However, maintenance of pregnancy on weeks 5 to 9 was associated to higher P_4 concentrations during this period (Starbuck *et al.*, 2004), which points to a late deficiency in P_4 concentrations in lactating dairy cattle. Other sources of LEM were also identified in high-yielding dairy cows (Grimard *et al.*, 2006).

5.3 – Plasma PSPB concentrations

Detection of PSPB at Day 7 in five cows (8%) reflected decaying concentrations from parturition (Humblot *et al.*, 1988). The detection rate of plasma PSPB and plasma PSPB concentrations of demi-embryo single pregnancies here reported are similar to those previously published for demi-embryo and whole-embryo pregnancies in dairy heifers (Lopes-da-Costa *et al.*, 2011). However, treatment with hCG, although increasing plasma P_4 concentrations had no effect on plasma PSPB concentrations. This contrasts to results reported in dairy heifer demi-embryo pregnancies supplemented with intravaginal P_4 (Lopes-

da-Costa *et al*,. 2011) and AI lactating dairy cows (Ayad *et al*., 2007). Therefore we can speculate that the enhanced embryo survival was not mediated by an increase in placental function but rather was mainly due to events occurring earlier at the maternal recognition period, as discussed above.

Deviation in placental secretion of PSPB, between single and twin pregnancies, occurred early in pregnancy (Day 35), and was sustained until the end of the study (Day 63). This trend was observed throughout all stages of pregnancies resulting from demi-embryo transfers (Dobson *et al*., 1993) and may reflect the number of trophoblastic cells present in the double placenta. However, in pregnancies resulting from *in vitro* produced embryos, plasma PSPB concentrations although also showing an early deviation between singles and twins (Day 40), did not maintain this trend throughout all stages of gestation (Vasques *et al*., 1995). Deviation between single and twin pregnancies on placental secretion of PSPB (Day 35) occurred earlier than deviation in embryonic size (unnoticed until Day 42). This is in accordance with anatomical data showing that increases in size and weight of the placenta occur earlier in pregnancy than those of the fetus (Eley *et al*., 1978).

In conclusion, here is reported a novel *in vivo* model considering a low developmental competence embryo (demi-embryo) and a sub-normal fertility recipient (high-yielding lactating dairy cow), as a tool to evaluate the effects and mechanisms of hCG treatment at ET on subsequent embryonic survival. This model can potentially be used to evaluate other therapeutic strategies designed for enhancing embryonic survival and the embryo-mother crosstalk. Treatment with hCG at ET significantly increased the survival rate of demi-embryos and the pregnancy rate of high-yielding lactating dairy cows. These effects, which were mediated by formation of secondary CL and the associated increase in plasma P_4 concentrations, were only observed until Day 25 of pregnancy. Embryos were rescued beyond maternal recognition of pregnancy, but later embryonic survival, growth until implantation and placental PSPB secretion until Day 63 of pregnancy were not affected by treatment or presence of secondary CL. Embryonic size at implantation was similar in single and twin pregnancies but PSPB secretion was higher in twin than in single pregnancies from Day 35 to 63, indicating that deviation in placental function occurred before deviation in embryonic size. Treatment with hCG may be of merit to decrease early embryonic loss in high-yielding lactating dairy cows and/or of low developmental competence embryos.

Chapter V

Evaluation of treatments with hCG and carprofen at embryo transfer in a demi-embryo and recipient virgin heifer model

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1 – Abstract

An *in vivo* model, combining a low developmental competence embryo (demi-embryo) and a high fertility recipient (virgin dairy heifer) was used to evaluate the effects of treatment with human chorionic gonadotrophin (hCG) and carprofen at embryo transfer (ET) on plasma progesterone (P_4) concentrations of recipients and on embryonic growth and survival. Embryos were bisected and each demi-embryo was transferred to a recipient on Day 7 of the estrous cycle. At ET, heifers (n = 163) were randomly allocated to treatment with hCG (2,500 IU im), carprofen (500 mg iv), hCG plus carprofen or to untreated controls. Plasma P_4 concentrations were measured on Days 0, 7, 14 and 21 of all recipients plus on Days 28, 42 and 63 of pregnant recipients. Pregnancy was presumed to be present in recipients with luteal plasma P_4 concentrations until Day 21 and confirmed by using transrectal ultrasonography on Days 28, 42 and 63. Embryonic measurements (crown-rump length and width) were obtained on Day 42. Treatment with hCG induced formation of secondary corpora lutea (CL) in 97% of heifers and increased ($P< 0.01$) mean plasma P_4 concentrations of non-pregnant recipients on Day 14 and of pregnant heifers on Days 14 to 63. This was associated to a significant decrease in early embryonic mortality. In contrast, subsequent embryonic losses resulted in a non-significant numerical increase by 8% of pregnancies maintained to Day 63. Therefore, treatment with hCG significantly rescued embryos through the maternal recognition of pregnancy window but was not able to support development thereafter. Treatment with carprofen at ET had no significant effects on plasma P_4 concentrations and rate of embryo mortality. Treatment with hCG plus carprofen at ET induced formation of secondary CL in 90% of heifers but decreased the luteotrophic effect of hCG, resulting in no effect on embryo survival. Low developmental competence embryos showed an intrinsic deficiency in overcoming the maternal recognition of pregnancy challenge and in proceeding to further

development until Day 28 of pregnancy, while mortality beyond this point was residual. Results on pregnancy rates should be confirmed in further experiments involving a larger sample size.

Keywords: demi-embryo; hCG; carprofen; progesterone; bovine

2 – Introduction

Pregnancy loss is a major cause of reproductive failure and of economic waste in dairy herds. Pregnancy losses are more prevalent (up to 40%) until maternal recognition of pregnancy (Humblot, 2001). Early and late embryonic losses are significantly greater in lactating cows than in heifers (Chagas e Silva *et al.*, 2002; Sartori *et al.*, 2002). This is related to the higher prevalence of sub-optimal post-ovulatory plasma progesterone (P_4) concentrations (CRRP NE-161, 1996; Chagas e Silva *et al.*, 2002) and to the lesser steroidogenic capacity of luteal cells (Shelton *et al.,* 1990; Pretheeban *et al.*, 2010) of cows, compared with heifers. Embryonic survival is dependent on the timely elongation and secretion of interferon tau by the blastocyst, in order to inhibit the luteolytic signal, features that are regulated by maternal concentrations of P_4 (Garrett *et al.*, 1988; Kerbler *et al.*, 1997; Mann & Lamming, 2001). This regulation by P_4 is apparently indirect (Clemente *et al.*, 2009) through the modulation of the luteolytic signal (Mann *et al.*, 1998) and the composition of the histotroph (Lonergan, 2011) and, probably by other embryo-maternal signaling pathways yet poorly understood or unknown (Bazer *et al.*, 2010).

Because of its role in pregnancy establishment and maintenance, P_4 supplementation of early pregnancy has received the focus of attention of practitioners and researchers, as a strategy to control embryonic mortality in cattle. Early luteal (Days 5 to 7) treatment with human chorionic gonadotrophin (hCG) induces ovulation of the dominant follicle, formation of secondary corpora lutea (CL) and increases plasma P_4 concentrations. These effects on embryo survival, however, have been controversial (recently reviewed by De Rensis *et al.*, 2010; Lonergan, 2011; Wiltbank *et al.*, 2011). Target treatment of risk populations seems to be of merit while general use of the drug seems to be redundant. This probably also applies to embryos. Survival of cryopreserved embryos was increased through hCG therapy at embryo-transfer (ET) but that of embryos originated through AI was not improved (Chagas e Silva & Lopes-da-Costa, 2005).

Another strategy to control embryo mortality is to inhibit the endometrial release of luteolytic and other pro-inflammatory prostanoids and cytokines, either at AI / ET or at the onset of maternal recognition of pregnancy, through the administration of non-steroidal anti-inflammatory drugs (NSAIDs). Results also have been controversial. Administration of flunixin meglumine at onset of maternal recognition of pregnancy (Days 13 to 16 post-AI) increased pregnancy rate in some (Guzeloglu *et al.*, 2007; Merril *et al.*, 2007), but not all experiments (Geary *et al.*, 2010; von Krueger and Heuwiesier, 2010; Rabaglino *et al.,* 2010). Flunixin meglumine administered at ET significantly increased pregnancy rate of recipients, although depending on location (Purcell *et al.*, 2005) and on embryo quality (Scenna *et al.*, 2005). Ibuprofen lysinate administered to recipient heifers one hour before ET significantly increased survival of frozen-thawed embryos (82% versus 56%, respectively), compared with controls (Elli *et al.*, 2001).

Carprofen is a long acting NSAID that allows treatment with a single administration and with a low milk excretion rate (Ludwig *et al.*, 1989), which is approved for use in lactating dairy cattle without a withdrawal period. In the bovine, a recent study showed that carprofen preferentially inhibits COX isoform 2 activity (Brentnall *et al.*, 2012). Treatment with carprofen had no positive effect on pregnancy rate, either given at AI in dairy cows (Heuwieser *et al.*, 2011) or at Days 14 to 16 post-AI in dairy heifers (von Krueger & Heuwieser, 2010). The effect of carprofen administered at ET on embryo survival has not been reported.

In this study, a novel *in vivo* model was used to evaluate therapeutic strategies designed to enhance embryonic survival. This embryo-recipient model (demi-embryo and virgin dairy heifer) allowed the evaluation of drug effects on the survival of low developmental competence embryos, ruling out main maternal factors (uterine health, sub-luteal P_4 concentrations) associated with embryo mortality. The objective of this study was to evaluate the effects of treatment with hCG, carprofen and hCG plus carprofen at ET on plasma P_4 concentrations of recipient virgin dairy heifers and on embryonic growth and survival. The main experimental hypothesis behind the study is that luteotrophic (hCG) and NSAID (carprofen) drugs, inducing changes in uterine environment and function, may rescue development and survival of low developmental competence embryos (demi-embryos). These effects on embryonic development and survival may occur in an additive or even synergic way following the simultaneous administration of hCG and carprofen.

3 – Material and Methods

3.1 – Recipients and treatments

The entire set of recipients was managed as a single group. Virgin Holstein heifers (n = 163) from a single dairy were enrolled in the experiment. These heifers were 15 months old, had a mean body condition score of 3 (scale 1 to 5), were healthy, normally cyclic and gynaecologically sound as evaluated by a routine veterinary examination. At this examination, heifers with a palpable mature CL were treated with a prostaglandin $F_{2\alpha}$ analogue (Veteglan, Laboratories Calier, Barcelona, Spain) and the tail head was painted to improve subsequent detection of estrus. All recipients were observed in standing estrus with a clear mucous vulvar discharge, and immediately before transfer, all had a single mature CL. At ET recipients were randomly allocated to one of four treatments: i) hCG (Chorulon, Intervet, Boxmeer, The Netherlands; 2,500 IU im); ii) carprofen (Rimadyl, Laboratories Pfizer, Portugal; 500 mg iv); iii) hCG plus carprofen; and iv) untreated controls.

3.2 – Embryo production, bisection and transfer

Embryos were recovered from superovulated Holstein donors from the same herd according to methods previously described (Chagas e Silva & Lopes-da-Costa, 2005). Quality grade 1 embryos (Stringfellow & Seidel, 1988) were selected for bisection as reported elsewhere (Lopes-da-Costa *et al.*, 2011). Briefly, one embryo at a time was washed in splitting medium (Vigro Splitting Medium, AB Technology, Pullman, WA, USA), moved into a 50 µl microdrop of the same medium and bisected through one microblade. Bisection was accomplished so that the two halves were of similar size and, for blastocysts so that the inner cell mass and the trophoectoderm cells were equally distributed in the two demi-embryos. Upon bisection, the two halves were transferred to culture medium (Vigro Holding Plus, AB Technology, Pullman, WA, USA) for 5 min, washed, evaluated, loaded in French mini-straws, and immediately transferred to the uterine horn ipsilateral to the CL bearing ovary of recipients on Day 7 of the estrous cycle (Day 0 = estrus). Demi-embryos were randomly allocated to the four treatments so that each donor produced one or more sets of demi-embryos for all treatments.

3.3 – Secondary CL and pregnancy evaluation

Presence of secondary CL was evaluated on Day 14 and pregnancy was evaluated on Days 28, 42 and 63 by ultrasonography, using a 7.5 MHz rectal linear probe. A positive pregnancy diagnosis relied on imaging of an embryonic vesicle with embryo proper and of embryonic/fetal heartbeat and movements. On Day 42 embryonic measures were taken, including the crown-rump length (CRL) and the longest embryonic width (made at mid body, at the stomach level; herein referred as width). These measurements were assessed twice, the resulting mean being recorded as the eligible measurement. Pregnancy was presumed in recipients with luteal plasma P_4 concentrations until Day 21. Four windows of embryonic/fetal mortality were defined: i) early embryonic mortality (EEM), occurring from Day 7 to Day 15; estimated to have occurred in heifers returning to estrus 18-24 days following the reference estrus (Day 0) and with low plasma P_4 concentrations (< 2.0 ng/mL) on Day 21; ii) late embryonic mortality 1 (LEM 1), occurring from Day 16 to Day 28; estimated to have occurred in heifers not returning to estrus on Days 18-24, with luteal plasma P_4 concentrations (> 3.0 ng/mL) on Day 21 and found non-pregnant on pregnancy diagnosis on Day 28; iii) late embryonic mortality 2 (LEM 2), occurring from Day 28 to Day 42 in heifers found pregnant on pregnancy diagnosis on Day 28 and subsequently found non-pregnant on Day 42; and iv) early fetal mortality (EFM), occurring from Day 42 to Day 63 in heifers found pregnant on pregnancy diagnosis on Day 42 and later found non-pregnant on Day 63.

3.4 – Blood collection and storage and plasma P_4 measurement

Blood samples from the caudal vessel were collected in all recipients on Days 0 (estrus), 7 (ET and treatment if any), 14, and 21. Pregnant heifers on Day 28 were further submitted to blood collection on Days 28, 42 and 63 of pregnancy. All blood collections were performed at 09.00 AM before feeding (except on Day 0). Blood was collected into 10 mL heparin containing syringes (NH_4-heparin, Monovette, Sarstedt) through an 18 G needle, immediately centrifuged at 400 X g for 20 minutes, and the supernatant plasma aliquoted into 1.5 mL cryotubes. These tubes were transported on ice to the lab and stored at -25ºC until assayed. Plasma P_4 concentrations were measured by a solid-phase without extraction chemiluminescent immunoassay in an IMMULITE 1000 analyzer (Siemens Healthcare Diagnostics, GmbH, Eschborn, Germany), using commercial kits (IMMULITE 1000

Progesterone Kit, Siemens Healthcare Diagnostics, Amadora, Portugal). The analytical sensitivity of the assay was 0.2 ng/mL. The inter-assay and intra-assay coefficients of variation were 12.5% and 7.1%, respectively.

3.5 – Statistical analysis

Data were analyzed using statistical software (Statistica for Windows, version 7.0, Statsoft, Tulsa, OK, USA, 2004). Categorical data were analyzed by the Fisher's exact test. Data from embryonic measurements were evaluated by analysis of variance (ANOVA). Data from plasma P_4 concentrations were also analyzed by ANOVA with repeated measures (7 days for pregnant and 4 days for non-pregnant recipients), considering the fixed effects of treatment, pregnancy (presence on Day 42), day (within-effect) and their interactions. Post-hoc LSD evaluations were computerized for all significant effects. Significance was tested at the 5% level ($P< 0.05$). Values are Mean ± SEM, unless otherwise specified.

4 – Results

Fourteen heifers were retrospectively removed from analysis because of the presence of luteal plasma P_4 concentrations on Day 0 (n = 8; 5%) and to noncompliance to the experimental protocol (n = 6). Treatments with hCG and hCG plus carprofen induced secondary CL in 36 of 37 (97%) and 34 of 38 (90%) heifers respectively, whereas treatment with carprofen did not induce the formation of secondary CL. Overall, secondary CL developed ipsilateral and contralateral to the primary CL on 46% and 54% of occasions, respectively. This distribution was similar in heifers treated with hCG alone or with hCG plus carprofen.

4.1 – Plasma P_4 concentrations

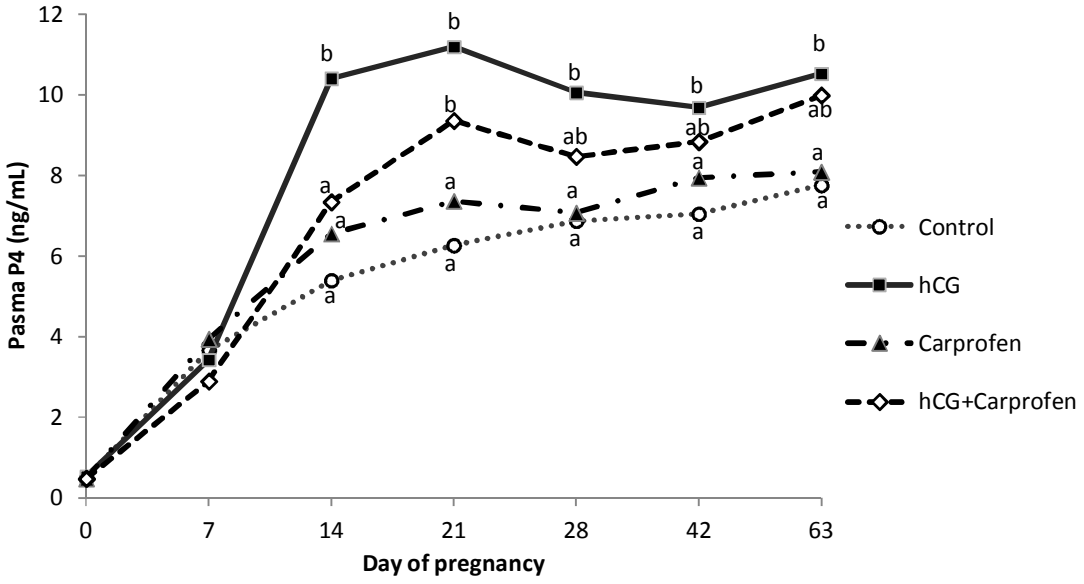
Group, pregnancy, Day and the interactions group by Day and pregnancy by Day affected ($P< 0.01$) plasma P_4 concentrations of recipients. Mean plasma P_4 concentrations on Day 7 were not affected by treatment and pregnancy (overall: 3.7 ± 0.1 ng/mL). Mean plasma P_4 concentrations on Day 14 were affected by treatment ($P< 0.01$), but not by pregnancy. Overall (pregnant and non-pregnant heifers), concentrations on Day 14 were similar in control and

carprofen treatments (6.1 ± 0.4 ng/mL and 6.6 ± 0.4 ng/mL, respectively), being lower than in hCG plus carprofen treatment (8.7 ± 0.4 ng/mL) and hCG treatment (10.4 ± 0.4 ng/mL). Concentrations of these latter two treatments differed.

Figure 20 illustrates mean plasma P_4 concentrations of recipients maintaining a pregnancy until Day 63. As shown, treatment with hCG increased mean plasma P_4 concentrations on Days 14 (compared with all other treatments) to 63 (compared with control and carprofen). Treatment with carprofen had no effect on mean plasma P_4 concentrations, whereas treatment with hCG plus carprofen only increased concentrations on Day 21 (compared with control and carprofen; but similar to hCG). Recipients presumed pregnant on Day 21 and later found non-pregnant on Day 28 (experiencing LEM1) had plasma P_4 concentrations until Day 21 similar to those of recipients pregnant on Day 28. In LEM1 heifers, the range of plasma P_4 concentrations on Day 21 was 4.1 to 16.6 ng/mL. Recipients pregnant on Day 28 and later found open on Day 42 (experiencing LEM2) had plasma P_4 concentrations until Day 28 similar to those of heifers maintaining pregnancy until Day 42.

Figure 20: Mean plasma P_4 concentrations of dairy heifers maintaining pregnancy until Day 63 following transfer of one demi-embryo and treatment with hCG or/and carprofen. Dots represent the LSD means; error bars are omitted to improve clarity. Dots with different superscript differ significantly: ab, P< 0.05.

Treatment groups: Control (n = 16); hCG (n = 19); Carprofen (n = 18); hCG + Carprofen (n = 17). Overall (Day 0 to Day 63) mean SE (range) of P_4 values: 0.14 (0.02 – 0.45)



ab, P< 0.05

4.2 – Embryonic size and survival

Embryonic size on Day 42 was not affected by treatments (P= 0.41; overall mean ± SD: CRL – 21.3 ± 2.0; width – 11.1 ± 1.2). Table 8 shows pregnancy rates on Days 21, 28, 42 and 63. Table 9 presents the rates of EEM, LEM1, LEM2 and EFM. The location of the secondary CL relative to the primary CL had no effect on pregnancy rate: overall, pregnancy developed on 56% of ipsilateral locations and on 51% of contralateral occasions.

Table 8: Pregnancy rates of dairy heifers following transfer of one demi-embryo and treatment with hCG or/and carprofen.

* Presumed pregnant on Day 21 = luteal plasma P_4 concentrations until Day 21 (range of values on Day 21: 4.1 to 16.6 ng/mL); ab, P< 0.05 (within columns)

Group	n	Pregnancy rate [n, (%)] on Day			
		21*	28	42	63
Control	37	20 (54.1)[a]	17 (45.9)	16 (43.2)	16 (43.2)
hCG	37	28 (75.7)[b]	21 (56.8)	19 (51.4)	19 (51.4)
Carprofen	36	23 (63.9)[ab]	19 (52.8)	19 (52.8)	18 (50.0)
hCG + Carprofen	38	25 (65.8)[ab]	19 (50.0)	17 (44.7)	17 (44.7)

Table 9: Embryo/fetal losses of dairy heifers following transfer of one demi-embryo and treatment with hCG or/and carprofen.

EEM: early embryonic mortality occurring from Day 7 to Day 15; estimated to have occurred in heifers returning to estrus 18-24 days following the reference estrus (Day 0) and with low plasma P_4 concentrations (< 2.0 ng/mL) on Day 21; LEM1: late embryonic mortality 1, occurring from Day 16 to Day 28; estimated to have occurred in heifers not returning to estrus on Days 18-24, with luteal plasma P_4 concentrations (> 3.0 ng/mL) on Day 21 and found non-pregnant on pregnancy diagnosis on Day 28; LEM2: late embryonic mortality 2, occurring from Day 28 to Day 42 in heifers found pregnant on pregnancy diagnosis on Day 28 and subsequently found non-pregnant on Day 42; EFM: early fetal mortality, occurring from Day 42 to Day 63 in heifers found pregnant on pregnancy diagnosis on Day 42 and later found non-pregnant on Day 63.

ab, P< 0.05; * P< 0.1 (tendency) (within columns)

Group	n	Embryo/fetal mortality – n (%)			
		EEM	LEM1	LEM2	EFM
Control	37	17 (46)[a]	3 (8)*	1 (3)	0 (0)
hCG	37	9 (24)[b]	8 (22)*	2 (5)	0 (0)
Carprofen	36	13 (36)[ab]	5 (14)	0 (0)	1 (3)
hCG + Carprofen	38	13(34)[ab]	7 (18)	2 (5)	0 (0)
Total	148	52 (35)	23 (16)	5 (3)	1 (0.7)

5 – Discussion

5.1 – Plasma P_4 concentrations

Treatment with hCG on Day 7 of the estrous cycle significantly increased plasma P_4 concentrations of non-pregnant heifers on Day 14 and, of pregnant heifers on Days 14 to 63. Interestingly, in pregnant heifers of group hCG plus carprofen, plasma P_4 concentrations on Day were lower than in group hCG, only reaching a similar mean to that of group hCG on Day 21. These results indicate that carprofen slowed and/or decreased the luteotrophic stimulus of hCG. This was not mediated through the efficiency of induction of secondary CL because in both treatments, all but one pregnant recipient developed secondary CL. As carprofen alone had no effect on plasma P_4 concentrations, simultaneous treatment with carprofen and hCG may impair post-ovulatory remodeling of the secondary CL, as observed in the rat with other NSAIDs (Gaytán *et al.*, 2006). This may compromise the subsequent luteal function of secondary CL, precluding or delaying full luteal expression of P_4 synthesis. Additionally, carprofen might have inhibited the release of PGE_2 in luteal and uterine cells, thus reducing the luteotrophic stimuli to the CLs. In fact, flunixin meglumin reduced the release of PGE_2 in an isolated bovine uterus model (Braun & Kietzmann, 2004). Altogether, this may reduce or even remove the potential beneficial effect of hCG on embryo survival.

Recipients experiencing LEM1 (mortality between Day 16 and Day 28) maintained luteal plasma P_4 concentrations (> 4 ng/mL) until Day 21, similar to those of heifers maintaining pregnancy until Day 28. Therefore, plasma P_4 concentrations until Day 21 did not allow the differentiation between LEM1 recipients and those maintaining pregnancy until Day 28. Also, recipients experiencing LEM2 (mortality between Day 28 and Day 42) had plasma P_4 concentrations until Day 28 similar to those of heifers maintaining pregnancy until Day 42. Again, this indicates that plasma P_4 concentrations until Day 28 were unable to distinguish between LEM2 recipients and those maintaining pregnancy until Day 42. Altogether, this indicates that in a high fertility recipient (virgin dairy heifer), embryonic mortality after maternal recognition of pregnancy cannot be attributed to a peripheral deficiency in P_4 concentrations until Day 28. In contrast, in a sub-fertility female model such as the lactating dairy cow, plasma P_4 concentrations following maternal recognition of pregnancy are related to the occurrence of late embryonic mortality (Stevenson *et al.*, 2008). This pinpoints a clear difference between heifers and lactating cows regarding the relevance of P_4 concentrations as a cause of late embryonic mortality.

5.2 –Embryonic survival

Pregnancy rate on Day 21 may be overestimated. Embryos dying after Day 16 trigger the maternal recognition of pregnancy mechanism and induce an extension of the estrous cycle with luteal plasma P_4 concentrations on Day 21 (Humblot, 2001). In addition, treatment with hCG *per se*, may induce an extension of the estrous cycle (Sianangama & Rajamahendran, 1992; Chagas e Silva & Lopes-da-Costa, 2005). Nevertheless, treatment with hCG decreased early embryonic mortality, thus increasing pregnancy rate on Day 21. This indicates that hCG treatment at ET rescued embryos through the maternal recognition of pregnancy window. In contrast, in hCG-treated heifers subsequent embryonic losses, mainly from Day 21 to Day 28, originated a Day 63 pregnancy rate that was no longer significantly different from that of controls. Nevertheless, compared to control, the numerical increase (8%) in pregnancy rate on Day 63 of hCG treatment can be of economic interest. At experiment design we estimated an increase in pregnancy rate in the range of 5-20% based on previous (Chagas e Silva & Lopes-da-Costa, 2005) and published (Nishigai *et al.*, 2001; Wallace *et al.*, 2011) results. Treatment with hCG induced the formation of secondary CL and a significant increase in plasma P_4 concentrations. Because almost all recipients developed secondary CL, the relative effects of presence of secondary CL and of increased plasma P_4 concentrations on embryo survival could not be dissociated.

NSAIDs inhibit the release of prostaglandins and other pro-inflammatory mediators through the inhibition of the COX-1 and COX-2 pathways. Uterine manipulation at ET induces the release of $PGF_{2\alpha}$ (Scenna *et al.*, 2005) and transfers requiring more manipulation of the uterus and putatively more traumatic significantly decrease pregnancy rate (Chagas e Silva *et al.*, 1999). This is probably due to a transient inflammatory state of the uterus, resulting in $PGF_{2\alpha}$ release, which is toxic for the embryo (Schrick *et al.*, 1993), and/or to an early demise of CL function. The reported beneficial effects on pregnancy rate following treatment with NSAIDs at ET (Elli *et al.*, 2001; Purcell *et al.*, 2005; Scenna *et al.*, 2005) were attributed to inhibition of prostaglandin release by the uterus in response to manipulation at transfer. Alternatively, NSAIDs could have induced an anti-inflammatory state on the uterus thus improving uterine receptivity. Carprofen, a long-acting NSAID, could potentially enhance the above effects and improve embryo survival. Treatment with carprofen at ET, although numerically increasing pregnancy rate on Day 63 by 7%, had no significant effect on pregnancy rate, compared with untreated controls. This is the first report on the use of carprofen at ET. In the present compromised embryo model, putative inhibition of manipulation-induced prostaglandin release by the uterus might have modestly enhanced embryo survival. This effect is unlikely

to have been the result of a uterine health promoting effect of the drug, considering the virgin state of the genital tract model used in the experiment.

Treatment with hCG plus carprofen was based on the rationale that induction of secondary CL and increase in plasma P_4 concentrations together with an inhibitory effect on manipulation-induced prostaglandin release at ET, could potentially originate an additive or even synergic effect on embryo survival. Carprofen apparently decreased the beneficial effect of treatment with hCG alone on embryo survival. This could be mediated through disruption of secondary CL function as discussed above.

The greatest prevalence of embryo losses occurred until Day 21 (overall 35%) and then from Day 21 to Day 28 (overall 16%), being losses in the late embryonic period and early fetal period only residual (3% and 0.7%, respectively). In the present *in vivo* model considering a high fertility recipient, this indicates that low developmental competence embryos have an intrinsic deficiency to overcome the maternal recognition of pregnancy window and continue development until Day 28. In accordance with these results, therapeutic strategies designed to enhance embryo survival should be attempted during early embryonic development. Treatment with hCG rescued embryo survival through the maternal recognition of pregnancy window but failed to support development thereafter. This might indicate that a second drug-induced embryonic stimulus placed during or shortly after maternal recognition of pregnancy could potentially enhance further embryonic survival. Further studies involving a larger population size are needed to evaluate the above issues and to confirm the effects of drugs (hCG and carprofen) on embryo survival.

Embryonic size at implantation is similar to that previously reported for demi-embryos (Lopes-da-Costa *et al.*, 2011) and was not affected by treatments. This indicates that the putative effects of treatments on early embryonic elongation and growth were no longer observed at Day 42. Early luteal treatment with P_4 or hCG induced longer conceptuses on Days 14 to 16 (Garrett *et al.*, 1988; Mann & Lamming, 2001), which probably also occurred in the present experiment. The fact that embryonic size at Day 42 of hCG-treated heifers was similar to that of untreated controls, reflects the plasticity of growth regulation mechanisms as previously reported (Lopes-da-Costa *et al.*, 2011).

In conclusion, herein is reported a novel *in vivo* model to evaluate therapeutic strategies designed to enhance survival of low developmental competence embryos. Treatment with hCG at ET induced formation of secondary CL and a significant increase in plasma P_4 concentrations, which was associated to a significant decrease in early embryonic mortality. However, subsequent embryonic losses resulted in a non-significant numerical increase by

8% of pregnancies established on Day 63. Therefore, treatment with hCG significantly rescued embryos through the maternal recognition of pregnancy window but was not able to support development thereafter. Alternatively, hCG could have simply delayed embryonic death and return to estrus, through its P_4 effect on maternal recognition of pregnancy. Treatment with carprofen at ET had no significant effect on plasma P_4 concentrations and rate of embryonic mortality. Treatment with hCG plus carprofen at ET induced formation of secondary CL but decreased the luteotrophic effect of hCG, resulting in no significant effects on rates of embryonic mortality. Poor developmental competence embryos had an intrinsic deficiency in overcoming the maternal recognition of pregnancy challenge and in proceeding to further development until Day 28 of pregnancy, whereas mortality beyond this point was only minimal. Therefore, therapeutic strategies designed to enhance survival of poor developmental competence embryos should be attempted before maternal recognition of pregnancy. Results on pregnancy rates should be confirmed in experiments involving a larger population size.

GENERAL DISCUSSION AND CONCLUSIONS

In ruminants, the establishment of pregnancy is dependent on a well-orchestrated sequence of events, resulting from a complex crosstalk between the mother and the developing embryo. This dialogue has been the object of extensive studies over the last decades, mainly because of the economic relevance of embryo-fetal losses in cattle production systems. Both steroids and prostanoids have been involved in the regulation of relevant reproductive events. However, the majority of studies have focused on their effects on reproductive female organs, while the role of these mediators in the developing embryo is poorly understood. Also, most of the studies evaluated the uterine-conceptus or the uterine-CL interactions. A potential and relevant crosstalk between the CL and the embryo is far from being fully explored. Finally, although a great effort has been made to develop and test anti-luteolytic strategies, the mechanisms underneath hormone action are still controversial. The present work considers both *in vitro* and *in vivo* studies designed to clarify the above questions.

The first *in vitro* experiment (Chapter I) was designed to develop a LC culture that could be used in co-culture with IVP embryos. As *in vitro* experiments considered the comparison between embryos co-cultured with LC and embryos cultured alone, it was initially relevant to test if LC could be cultured under embryonic requirements, such as simple media (Holm *et al.*, 1999), serum concentrations only up to 5% (Holm *et al.*, 1999; Rizos *et al.*, 2003), a hypoxic atmosphere (5%; Yuan *et al.*, 2003), and a 38.5°C to 39°C incubation temperature (Wang *et al.*, 1991), without significant impairment of LC function. Restrictive media as SOF may not provide specific requirements of somatic cells, with consequential loss of their *in vivo* morphological and functional properties, which in turn may affect their putative embryo-regulatory properties (Reischl *et al.*, 1999). Theoretically, early CL stage luteal cells would be the elected stage for our co-culture due to their synchrony with early developing IVP embryos. Results unequivocally demonstrated that these early luteal cells showed the best adaptation to embryonic *in vitro* culture conditions, expressing the highest steroidogenic potential in restrictive media and low oxygen tension atmospheres. This latter feature was however somehow expected, as early luteal cells develop in a low *in vivo* hypoxic environment (Nishimura & Okuda, 2010). Also, although deprival of serum was reported to cause growth arrest accompanied by cell death in rat luteal cells (Goyeneche *et al.*, 2006), in the present study serum concentrations optimal to embryo IVP were shown to support luteal growth and function.

A second relevant conclusion of this work was that bovine luteal cells can be cryopreserved without affecting subsequent cell viability and function as evaluated by P_4 and PGs production. This enabled the use of pools of frozen–thawed cells in the following experiment, thus decreasing the variation of cell function associated with primary cell cultures. As a final conclusion of this experiment, frozen-thawed early luteal cells maintain their growth, viability and function under *in vitro* culture conditions required for early embryo culture, enabling the use of these cells for studying the steroidogenic and prostanoid interactions in an embryo co-culture model.

Chapter II and III i*n vitro* experiments demonstrated that bovine early embryos already transcribe genes coding for enzymes of the PGs and P_4 synthesis pathways (PTGS2, PGFS, PGES, StAR, $P450_{scc}$ and 3β-HSD), and produce P_4, $PGF_{2\alpha}$ and PGE_2 into culture medium. These were the first reports on P_4 and $PGF_{2\alpha}$ production by pre-hatching bovine embryos. As receptors for P_4 and $PGF_{2\alpha}$ were described in the bovine blastocyst (Clemente *et al.*, 2009; Scenna *et al.,* 2006), an autocrine role for these mediators in embryonic development is suggested. PGs were implicated in blastulation and hatching in several species (mouse: Baskar *et al.*, 1981; Chida *et al.,* 1986; Huang *et al.,* 2004; ovine: Sayre & Lewis, 1993; goat: Sayre, 2007; bovine: Pereira *et al.,* 2005b). El-Sayed *et al.* (2006) performed large-scale transcriptional analysis of bovine embryo biopsies and related results to subsequent pregnancy outcome following transfer to recipients (3 groups: no pregnancy, reabsorbed embryos and calf delivery). Authors found that biopsies from embryos that resulted in a calf delivery had a higher transcription of PTGS2 than biopsies from the other two groups. Additionally, as PRs are present in the bovine oviduct (Kenngott *et al.*, 2011), uterus (Robinson *et al.,* 2001; Okumu *et al.,* 2010), CL (Rueda *et al.,* 2000), and blastocyst (Clemente *et al.,* 2009), P_4 synthesized by the embryo may exert autocrine and/or paracrine actions in the surrounding endometrium, which may be relevant for inducing local uterine receptivity.

The analysis of transcription levels of genes coding for enzymes of the P_4 and PGs synthesis pathways revealed significant differences between embryonic stages of development. The first cellular differentiation (from blastomeres to ICM and TE cells) within the developing embryo was associated with an increase in transcription levels of all except the StAR gene. In fact, transcripts of $P450_{scc}$ and 3β-HSD were even absent in some replicates of CM and YBL, which suggests that these two genes start their transcription at the time blastomeres differentiate into the ICM and TE cells. This also prompts for a role of PGs in blastocoel expansion and hatching, and for a paracrine role of both PGs and P_4 in the early embryo-maternal crosstalk. Recent studies used the Affymetrix microarray platform to identify

differences between the endometrial transcriptome of pregnant and cyclic heifers (Gao *et al.*, 2009; Forde *et al.*, 2010). In these studies, differences between the endometrial transcriptome of pregnant and cyclic heifers were only detected at Day 15 or 16, i.e. at onset of MRP. However, using new RNA sequencing technology, several genes had differentiated transcription in pregnant and cyclic endometria as early as Day 13 (Forde *et al*., 2012). In our study, the fact that early embryos already had active transcription of several genes of the steroidogenic and prostanoid metabolic pathways, and already produced their end-point products, may indicate that these early embryonic-derived factors induce subtle and localized, however potentially relevant autocrine and paracrine changes in the endometrium, resulting in enhanced embryonic development and uterine receptivity. These changes seem to be independent of IFN-τ action, as they occur well before the reported onset of IFN-τ activity.

The influence of oocyte donor age (pre-pubertal (PP) versus post-pubertal cyclic (C)) on transcription levels of genes coding for P_4 and PGs synthesis pathways was evaluated. Literature reports showed that embryos derived from PP oocyte donors had altered kinetics of development (Ptak *et al.*, 1999; Majerus *et al.*, 2000; Leoni *et al.*, 2006a) lower *in vivo* survival (O'Brien *et al.,* 1997; Ptak *et al.*, 1999; 2003; Kelly *et al.,* 2005) and changes in the expression of developmentally important genes (cattle: Oropeza *et al.*, 2004; sheep: Leoni *et al.*, 2006b). However, results here presented did not reveal a significant effect of oocyte donor age on transcription levels of genes coding for enzymes of the PGs and P_4 synthesis pathways. This may be related to the use of older PP oocyte donor heifers (age range: 6-9 months), as full developmental competence of PP oocytes was suggested to be achieved at the age of 7 to 8 months, which is close to onset of puberty in *Bos taurus* breeds (Camargo *et al.*, 2005). However, 3β-HSD transcript levels tended to be higher in embryos from C than from PP heifers, which may indicate a lower P_4 production ability of PP donor derived embryos, potentially associated to the above reported lower *in vivo* survival rate and disturbances in the kinetics of development.

Co-culture with LC induced an embryotrophic effect, significantly increasing blastocyst yield and quality. Luteal cells produced P_4, PGE_2 and $PGF_{2\alpha}$, and a direct positive effect of P_4 (Merlo *et al.*, 2006; Ferguson *et al.*, 2012) and PGE_2 (ovine: Sayre & Lewis 1993; caprine: Sayre, 2007) on *in vitro* embryo development was reported. However, co-culture with LC had no effect on embryonic mean cell number, gene transcription and production of PGs and P_4. It is therefore possible that the embryotrophic effect induced by co-culture with LC might have been due, at least partially, to a typical homeostatic somatic cell effect. This effect may be generated through the production of other embryotrophic factors and/or through the removal

of toxic products of embryonic catabolism, protection against oxidative stress and modulation of medium physico-chemical properties (reviewed by Orsi & Reischl, 2007).

On the other hand, embryos did not exert a luteotrophic effect upon co-cultured LC, in terms of P_4 and PGs production. This observation corroborates results from previous *in vivo* work of our team. Comparing plasma P_4 profiles of non-bred, AI-bred and ET-bred virgin dairy heifers, no significant luteotrophic effect of embryonic origin was detected until onset of MRP (Chagas e Silva & Lopes-da-Costa, 2005). However, it is unlikely that this putative luteotrophic effect of embryonic origin, independent of IFN-τ mechanism, could be detected at the systemic circulation. Data from our *in vitro* experiments indicate that, if direct interactions between the CL and the embryo occur early in pregnancy, they are not modulated through P_4, PGE_2 and $PGF_{2\alpha}$. In fact, due to the reduced amounts of these mediators detected in culture medium it is more plausible that their action is exerted in an autocrine or uterine paracrine fashion.

Oil overlaying is routinely used in embryo culture to prevent dehydration, pH variation, and accumulation of toxic components (Tae *et al.*, 2006). In the present work, oil overlaying of culture wells increased blastocyst yield and quality, although there was no additive embryotrophic effect following the use of co-culture and oil overlaying. However, oil overlaying induced a 50-fold decrease in P_4 quantification in culture medium. This was likely due to the lipophilic nature of this hormone, allowing diffusion from the aqueous to the oil fraction, as previously reported (Shimada *et al.,* 2002; Clemente *et al.*, 2009). Therefore, measurement of P_4 concentrations in the aqueous and oil fractions may be necessary, especially if the expected steroid concentrations are low. Also, this effect needs to be taken in consideration when designing *in vitro* experiments where the stimulation of cells or embryos by steroids is to be evaluated, as results can be biased by depletion of those compounds from media.

In the present thesis, two *in vivo* models were used to evaluate the effects and mechanisms of therapeutic strategies designed to enhance embryonic survival followingt ET (Chapter IV and V). The first *in vivo* model considered a low developmental competence embryo (demi-embryo; Chagas e Silva & Lopes-da-Costa, 2005) and a sub-normal fertility recipient (high-yielding lactating dairy cow; Lucy, 2001; Rodriguez-Martinez *et al.*, 2008). Controversial results arising from hCG therapy were related to systematic treatment rather than treatment of target females. High-yielding lactating dairy cows, due to sub-normal plasma P_4 concentrations, are a group at risk that may potentially benefit from P4 supplementation treatments (Santos *et al.*, 2001). Also, compromised embryos may also benefit from exogenous P_4 stimulation (Chagas e Silva & Lopes-da-Costa, 2005). This model allowed the

evaluation of drug effects on the worst scenario. The second *in vivo* model combined a low developmental competence embryo (demi-embryo; Chagas e Silva & Lopes-da-Costa, 2005) and a high fertility recipient (virgin dairy heifer; Chagas e Silva *et al.*, 2002b). This model allowed the evaluation of drug effects on the survival of compromised embryos, ruling out main maternal factors (uterine health, sub-luteal P_4 concentrations) associated to embryonic mortality.

In Chapter IV, treatment with hCG at ET significantly increased the survival rate of demi-embryos and the pregnancy rate of high-yielding lactating dairy cows. These effects were mediated by formation of secondary CL and the associated increase in plasma P_4 concentrations. However, stimulation of primary CL without formation of secondary CL had no effect on embryo survival, suggesting that secondary CL may act locally by mechanisms other than P_4, or in association with P_4. The beneficial effects of hCG were only observed until Day 25 of pregnancy. Therefore, embryos were rescued beyond MRP, but later embryonic survival was not affected by treatment. This observation was confirmed in the second *in vivo* model (Chapter V), where treatment with hCG at ET in virgin dairy heifers was also associated to a significant decrease in early embryonic mortality, but had no effect thereafter. Early pregnancy high circulating P_4 concentrations stimulate conceptus growth, elongation and secretion of IFN-τ, thus potentially enhancing survival beyond MRP (Rizos *et al.*, 2012). Altogether, this indicates that development beyond MRP is not under strict dependency on P_4 concentrations. In fact, in both *in vivo* experiments, plasma P_4 concentrations until Day 25 to 28 were similar in recipients with late embryonic mortality and recipients maintaining pregnancy until implantation. As treatment with hCG had no effect on embryonic growth until implantation and on plasma PSPB concentrations until establishment of placentation, the hCG-induced enhanced early embryonic survival reported in Chapter IV (and possibly that reported in Chapter V) was not mediated by an enhanced growth until implantation or an enhanced placental function, but was connected to events occurring earlier, at MRP.

In Chapter IV, embryonic size at implantation and PSPB secretion were also evaluated. This allowed a description of PSPB profiles during single and twin demi-embryo early pregnancies. Embryonic size at implantation was similar in single and twin pregnancies and was not affected by hCG treatment. Previous studies showed that fetal weight was similar in single and multiple pregnancies at Day 56 (Echternkamp, 1992) and at Day 95 (Bellows *et al.,* 1990) of pregnancy, but was higher in single than in multiple fetuses at Day 180 (Bellows *et al.,* 1990). Therefore, deviation in size between singletons and twins occurs late in pregnancy, but the exact moment of this deviation is unknown. Deviation in placental secretion of PSPB,

between single and twin pregnancies, occurred early in pregnancy (Day 35), and was sustained until the end of the study (Day 63), which corroborates previous data (Dobson *et al.*, 1993; Vasques *et al.*, 1995), and was possibly connected to an increase in the number of trophoblastic cells present in the double placenta. Therefore, the deviation between single and twin pregnancies on placental secretion of PSPB (Day 35) occurred earlier than the deviation in embryonic size (unnoticed until Day 42). This discrepancy is supported by anatomical data showing that increases in size and weight of the placenta occur earlier in pregnancy than those of the fetus (Eley *et al.*, 1978).

In Chapter V, the effect of treatment at ET with carprofen, a long acting NSAID, was evaluated. Carprofen, when used alone, had no significant effects on plasma P_4 concentrations and prevalence of embryonic mortality. Putative beneficial effects on pregnancy rate following treatment with NSAIDs at ET have been attributed to an inhibition of PG release by the uterus in response to manipulation at transfer (Elli *et al.*, 2001; Purcell *et al.*, 2005; Scenna *et al.*, 2005). However, it seems that if uterine manipulation at ET is minimal and ETs are performed by a trained technician, the use of a NSAID is redundant. This was probably the case of this experiment using a virgin heifer recipient model, which also very likely presents a healthy uterus.

Treatment with hCG plus carprofen at ET induced the formation of secondary CL, but carprofen slowed and/or decreased the luteotrophic stimulus of hCG. This observation evidences the role of PGs on ovulation and luteal development. In rabbits and women PTGS2 inhibition induced luteinized unruptured follicles, sometimes associated to a delay in the rise of P_4 and lower P_4 concentrations in the first half of the luteal phase (Killick & Elstein, 1987; Smith *et al.*, 1996; Pall *et al.*, 2001; Salhab *et al.*, 2001, 2003). Carprofen might have annulled the luteotrophic actions of luteal and uterine PGE_2 in the CLs (Braun & Kietzmann, 2004). Therefore, as observed in this experiment, the administration of carprofen can decrease or even annul the beneficial effect of hCG on embryo survival. However, it is still not clear whether the use of anti-luteolytics can in some situations enhance embryonic survival, as results of several studies have been controversial.

As an overall conclusion, work presented in this thesis evidenced that pre-hatching bovine embryos are able to produce P_4 and PGs that may have a relevant autocrine role in embryo development (blastulation and hatching) and/or paracrine role in the uterus, stimulating uterine receptivity. An embryotrophic effect of luteal cells origin was evidenced, but this was not associated with a change in embryonic P_4 and PGs production. In contrast, a luteotrophic

effect of embryonic origin was not detected, which indicates that steroidogenic and prostanoid mediated embryo-maternal crosstalk is directed primarily to the uterus. *In vivo* treatment with hCG induces formation of secondary CL, increases plasma P_4 concentrations and embryonic survival beyond MRP. However, further development to implantation and placental function are not affected, which may indicate that survival beyond MRP is not linked to maternal P_4 concentrations. Carprofen treatment at ET had no effect in the healthy virgin heifer uterus scenario, and partially reverted the luteotrophic effect of hCG.

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