Solving the puzzle of inter-embryonic communication in bovine embryo group culture by proteomics.

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I have no special talents.

I am only passionately curious.

(Albert Einstein)

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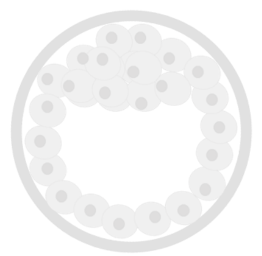
LIST OF ABBREVIATIONS

2D-PAGE	2D-polyacrylamide gel electrophoresis
ACN	Acetonitrile
ACR	Apoptotic cell ratio
ART	Assisted reproductive techniques
BAD	Bcl2-associated death promoter
BAX	Bcl2-Associated X
BFA	Brefeldin A
Blast	Blastocyst
BSA	Bovine serum albumin
CATH	Cathepsin-L
COCs	Cumulus cell-oocyte complexes
CPEPB	Cytoplasmic polyadenylation element binding protein
CREB	cAMP response element-binding protein
CSF-1	Colony stimulating factor-1
CSF-R	Colony stimulating factor-1 receptor
dpi	Days post insemination
EB	Expanded blastocyst
EGA	Embryonic genome activation
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FGF-R	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FP	Fertility preservation
GCPR	G-coupled protein receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GM-CSF R	Granulocyte-macrophage colony-stimulating factor receptor
HA	Hyaluronic acid
HB	Hatching or Hatched blastocyst
hpi	Hours post insemination

HPLC	High performance liquid chromatography
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IFN	Interferon
IGF	Insulin-like growth factor
IGF-R	Insulin-like growth factor receptor
IL	Interleukin
iTRAQ	Isobaric tags for relative and absolute quantitation
ITS	Insulin, transferrin and selenium
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
IVP	*In vitro* production
JNK	c-Jun N-terminal kinase
kDa	kilo-Dalton
LC	Liquid chromatography
LIF	Leukemia inhibitory factor
LIF-R	Leukemia inhibitory factor receptor
LOS	Large offspring syndrome
LPA	Lysophosphatidic acid
MDC	Membrane damaged cell
MDM2	Murine double minute 2
MMTS	S-methylmethan-thiosulfate
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
NB	Normal blastocyst
NC	Non-cleaved embryo
OHSS	Ovarian hyper stimulation syndrome
OPU	Oocyte pick-up
OR	Odds ratio
PAF	Platelet activating factor
PDGF	Platelet-derived growth factor
PDGF-R	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol-4,5,-bisphosphate 3-kinase
PBS	Phosphate buffered saline
PDK1	3-phosphoinositide-dependent protein kinase 1
PG	Prostaglandin

PIF	Preimplantation factor
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PN	Pronuclei
PPAR	Peroxisome-proliferator-activated receptor
PVP	Polyvinylpyrrolidone
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RTK	Tyrosine kinase receptor
RXR	Retinoid X receptor
S1P	Sphingosine-1 phosphate
SCF	Stem cell growth factor
SCF-R	Stem cell growth factor receptor
SELDI-TOF MS	Surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry
s.e.m.	Standard error of the mean
sHSA-G	Soluble human leucocyte antigen-G
SOFaa	Synthetic oviductal fluid supplemented with essential and non-essential amino acids
TALP	Tyrode's medium supplemented with albumin, sodium lactate and sodium pyruvate
TCM	Tissue culture medium
TCN	Total cell number
TE	Trophectoderm cells
TEABC	Triethylammonium bicarbonate
TGF	Transforming growth factor
TGF-β R	Transforming growth factor beta receptor
TNF	Tumour necrosis factor
TNF-R	Tumour necrosis factor receptor
TR	Embryos that can be transferred on day 3
TUNEL	Terminal deoxynucleotidyl transferase-dUTP nick end labelling
UT	Embryos that can be used for uterine transfer or cryopreservation
UPS	Unconventional protein secretion
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor
WOW	Well-of-the-Well
YB	Young blastocyst
ZP	Zona pellucida



CHAPTER 1

GENERAL INTRODUCTION

Adapted from

Autocrine embryotropins revisited: how do embryos communicate with each other *in vitro* when cultured in groups?

Wydooghe E, Vandaele L, Heras S, De Schauwer C, De Sutter P, Deforce D, Peelman L and Van Soom A. Biological Reviews. 2017. 96 (6), 1181–1196.

1. A little history

Already more than a century ago, scientists realized that knowledge of mammalian fertilisation would increase if the process could be observed under the microscope *in vitro*. Towards that end, scientists started working on an *in vitro* fertilisation (IVF) system, at the beginning mainly with eggs of sea urchin. It was in the 1950s, with the recognition of the phenomenon of sperm capacitation (Austin 1952, Chang 1951) that the first repeatable technique for fertilizing mammalian eggs *in vitro* became available (Chang 1959), using sperm recovered from the rabbit uterus 12 hours after mating. Another milestone in the development of IVF was the work of Yanagimachi (1969), in which he was able to demonstrate sperm capacitation *in vitro* in the hamster. Based on these data, Edwards was able to fertilize the first human oocyte *in vitro* in 1969 and almost 10 years later, in 1978, Louise Brown, the first human "test tube"-baby, was born (Edwards, et al. 1980). In parallel with the work on human IVF, an increasing amount of efforts was dedicated to establish an *in vitro* production system for bovine embryos. In 1981, the first *in vitro* calf, called 'Virgil', was born after the *in vitro* fertilisation of an *in vivo* matured oocyte by Brackett et al. (1982) and only in 1990 the first calf was born out of an *in vitro* produced blastocyst, starting from an immature oocyte (Fukuda, et al. 1990, Lu, et al. 1987).

2. Preimplantation development of embryos in their natural habitat

In vitro production of embryos (IVP) implies an extraordinary change in the environment where the beginning of a new organism takes place. The natural environment allowing normal embryonic development to occur is the female genital tract: fertilisation and the first cleavages of the embryo take place in the oviduct. Subsequently the embryo will enter the uterus, where implantation occurs. Already at this very beginning of pregnancy, a specific interaction between the mother and embryo is necessary, in order to create the optimal environment for early embryo development, implantation, maintenance of pregnancy and the future health of the offspring.

However, i*n vivo*, mammalian embryos in the reproductive tract are exposed to a great many autocrine, paracrine and endocrine factors. Autocrine factors are produced and released by the embryo itself and act upon the embryo or the neighbouring embryos (O'Neill 2008b). Paracrine factors play an important role in the embryo-maternal dialogue, they can originate from cells of the reproductive tract and have an effect on the embryo, or can be secreted by the embryo and have an effect on the oviduct or uterus. Both preimplantation embryos and the female genital tract secrete growth factors and they can respond to these growth factors, since their corresponding receptors are present in order to communicate (see supplementary table 1 & 2). Besides growth factors, it has

recently been shown that also extracellular vesicles play a crucial role in embryo-maternal communication (for review see, Burnett, et al. 2016; Pavani, et al. 2017). Extracellular vesicles carry and transfer regulatory molecules, such as microRNAs, mRNAs, lipids and proteins, as a consequence paracrine factors might be very diverse in nature. Endocrine factors, such as insulin are released by distant cells in the blood or lymph stream and may form part of the luminal fluid of the reproductive tract (O'Neill 2008b). In the next paragraphs, some examples of endocrine and paracrine communication *in vivo* will be discussed, but since autocrine communication has been mainly examined *in vitro*, autocrine factors will be discussed in depth in the remaining part of the introduction.

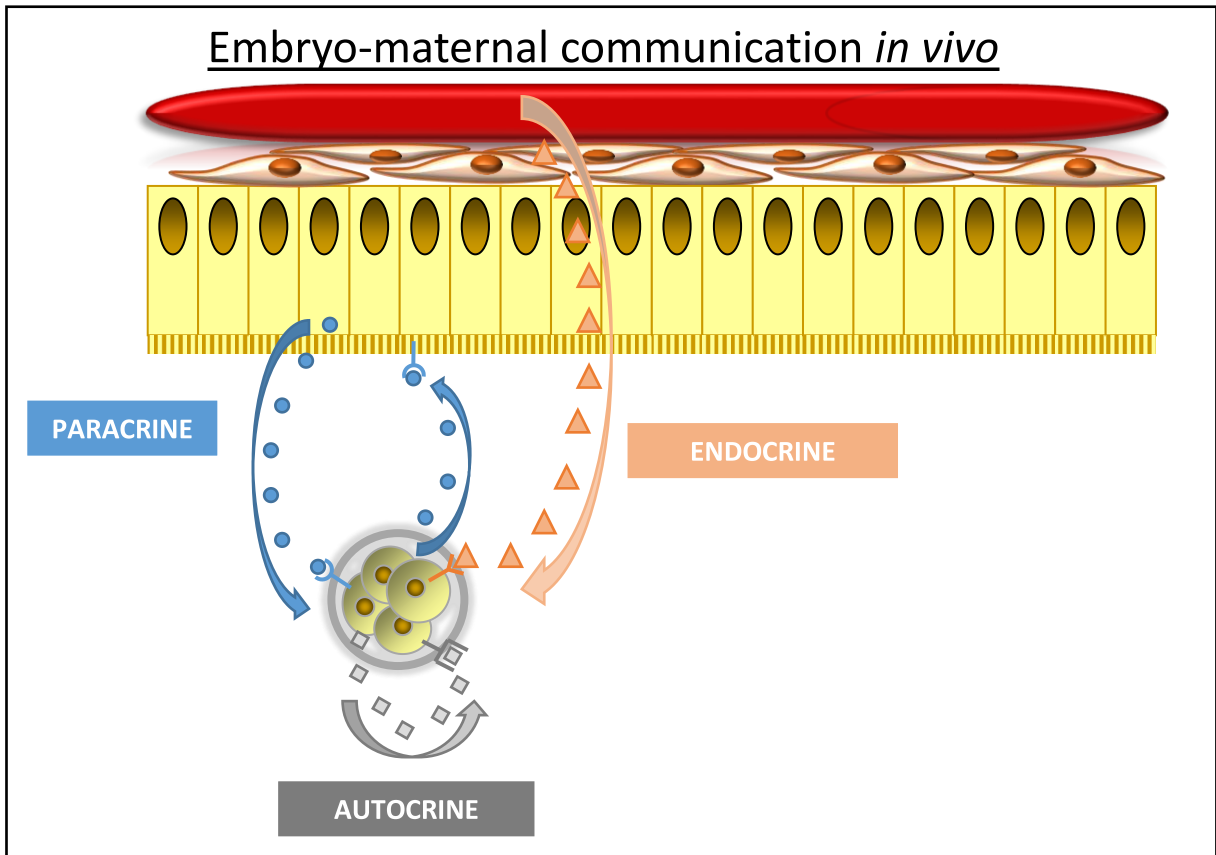


Figure 1. *Communication between embryo and maternal tract in vivo involve paracrine, autocrine and endocrine factors.*

Endocrine signalling factors

Long range signalling molecules, such as insulin, oestrogens and progesterone, have been identified as being important in embryo-maternal communication (Fazeli and Pewsey 2008). Insulin is a peptide hormone that is synthesized and secreted by pancreatic β-cells in response to propionate, butyrate and glucose (Harmon 1992). Insulin contributes to the energy supply of the endometrium, by facilitating glucose uptake (Fornes, et al. 2010), which is the indispensable energy source for the

endometrial gland activity i.e. secretion of paracrine signalling factors. In women with polycystic ovary syndrome, which is characterized by high androgen levels and hyperinsulinemia, glucose supply at the endometrium is disturbed and is contributing, at least partially, to reproduction failure in these women (Homburg 2006). Recently, it has been shown that insulin up-regulates gene and protein expression of the pro-angiogenic factor PROK1. PROK1 dysfunction disturbs the migration of endometrial stromal cells and migration and invasion of trophoblasts, essential for successful implantation (Dorina, et al. 2018). Besides an effect on the uterus, insulin might also have an influence on the embryo directly since the presence of an insulin-receptor has been shown in oocytes, cumulus cells (Purcell, et al. 2012) and in embryos from the zygote to the blastocyst stage (Schultz, et al., 1992). Limited knowledge is available concerning the direct effect on insulin on embryo development *in vivo*, however insulin has been used for many years as a stimulatory factor for *in vitro* embryo production (for review see, Laskowski, et al. 2016).

Steroid hormones, such as oestrogens and progesterone are produced from cholesterol in the ovary and reach the genital tract via the general circulation or directly with the follicular fluid at ovulation (Belin, et al. 2000). It is generally accepted that progesterone plays a crucial role in the establishment and maintenance of pregnancy through its action on the uterine endometrium. Up till now, there is no consensus concerning the direct effect of progesterone on preimplantation embryos. Clemente et al. (2009) showed that progesterone cannot directly support blastocyst elongation, interferon-tau production or maternal recognition of pregnancy. However, its effect is indirect, with high progesterone concentrations in blood and uterus triggering changes in the uterine environment, leading to changes in histotrophe composition which are facilitating embryo elongation and interferon-tau production. The indirect effect of progesterone on early embryo development has also been confirmed by Pereira and co-workers (2009). Larson *et al.* (2011) did not observe a direct positive effect of progesterone supplementation to *in vitro* culture medium in terms of embryo development. This is in contrast with Ferguson *et al.* (2012), who observed a beneficial effect of progesterone supplementation (starting from day 3 of culture) on blastocyst development and quality.

Paracrine signalling factors

Short range signalling molecules which are involved in the communication between embryo and genital tract are less defined and may involve enzymes, cytokines, growth factors, angiogenic factors, apoptotic factors, and adhesion molecules (Hill 2001), see also supplementary table 1 en 2. In maternal-embryonic communication, two kinds of paracrine signalling factors can be identified: factors secreted by the oviduct or uterus and acting on the embryo, and factors secreted by the embryo

proper, which interact with the oviduct or uterus. The analysis of these compounds is especially difficult since some of them are very short-lived, can only be detected for a very brief period of time or at a very low concentration.

In the horse, for example, paracrine embryo–oviduct interactions may underlie the differential transport of fertilized and unfertilized oocytes in the oviduct. It has been demonstrated that prostaglandin E2 (PGE2) is released by viable horse embryos but not by unfertilized oocytes or degenerate embryos. The oviduct smooth muscle relaxes at the level of the uterotubal papilla in the presence of a viable equine embryo, hastening oviductal transport of the embryo, and opening the uterotubal papilla in order to allow the embryo to enter the uterus (Weber, et al. 1991a, b). Unfertilized oocytes or degenerate embryos are not able to enter the uterus. Likewise, lysophosphatidic acid (LPA) has been reported to be involved in bovine embryo–endometrium interactions: both the preimplantation embryo and endometrium are able to secrete LPA, and the corresponding receptor has been detected in the pregnant endometrium (Woclawek-Potocka, et al. 2009). By stimulation of PGE2 synthesis and inhibition of PGF2α synthesis, the LPA pathway maintains pregnancy in cows by supporting the function of the corpus luteum (Woclawek-Potocka, et al. 2010).

3. Preimplantation development of embryos *in vitro*

3.1 Standard *in vitro* embryo production protocol in research labs

In vitro production (IVP) of bovine embryos is a three-step process involving *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and *in vitro* culture (IVC). In this thesis, all oocytes were collected from bovine ovaries obtained from a local slaughterhouse. Follicles between 2 and 8 mm diameter were punctured and the obtained immature oocytes were subsequently allowed to mature *in vitro* in groups of 60 oocytes in 500 µl TCM-199 maturation medium at 38.5°C during 22 hours in order to complete nuclear and cytoplasmic maturation. Nuclear maturation involves resumption of meiotic division, from the germinal vesicle stage up to metaphase II with the extrusion of the first polar body. Cytoplasmic maturation on the other hand requires the accumulation of mRNA, proteins, substrates and nutrients that are necessary during fertilisation and early cleavages of the embryo (Watson 2007). During IVM, oocytes are incubated in TCM-199, commonly supplemented with energy sources (e.g. pyruvate), growth factors (e.g. EGF), hormones (e.g. gonadotrophins) and/or a complex mixture as foetal bovine serum (FBS). Serum was typically added during the IPV process to supply growth factors and energy sources, however lately the use of serum-free, defined media is apparent in animal IVP.

The following step in IVP is fertilisation of mature oocytes by capacitated bovine sperm. Capacitation is a series of biochemical and physiological modifications in order to render them competent to fertilize an oocyte (Ickowicz, et al. 2012). Most *in vitro* bovine production systems make use of frozen-thawed semen. In our IVP system, after thawing, sperm is loaded on a discontinuous Percoll density gradient in order to select viable, motile and morphologically normal bull spermatozoa (Mortimer 1994, Thys, et al. 2009). By centrifugation, spermatozoa with good nuclear morphology will equilibrate in the higher-density fraction of the gradient since they are denser than inferior spermatozoa (Gordon 2003). After this selection step, mature oocytes are co-incubated with the viable spermatozoa (often at a concentration of 1 x 10^6 sperm cells/ml), in capacitating medium.

After about 20 - 24 h of co-incubation, presumed zygotes are vortexed for 3 minutes to remove the excessive sperm and cumulus cells, before they are transferred to culture medium. The embryos are cultured in groups of 25 in a droplet of 50 µl medium synthetic oviductal fluid supplemented with essential and non-essential amino acids (SOFaa) and 5% FBS, covered by mineral oil and incubated at 38.5°C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. At 8 days post insemination (dpi), 30 to 40 % of the embryos have reached the blastocyst stage.

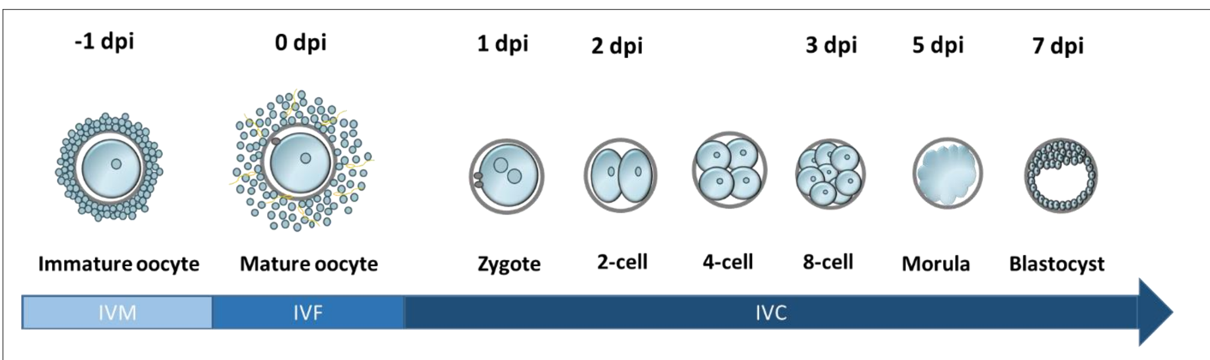


Figure 2: *Overview of in vitro embryo production (dpi = days post insemination).*

3.2 Inter-embryonic communication in group culture

In the absence of the maternal genital tract, preimplantation embryos can develop *in vitro* in culture medium where all communication with the oviduct or uterus is absent. *In vitro*, preimplantation embryos can develop relatively easy and are able to develop to the hatched blastocyst stage in very simple culture media (Biggers 2002, Block and Hansen 2007). Based on these observations, it was assumed that early embryonic development could occur in the absence of signalling factors, in contrast to the situation in somatic cells in which signalling factors are essential as mitogens to induce cell division and as survival factors to avoid somatic cells entering the apoptotic pathway. However, recent evidence has shown that preimplantation embryos also require signalling

factors that modulate cell growth and cell division, or that have anti-apoptotic functions (O'Neill, et al. 2012). It has been shown that co-culture of the embryo with somatic cells as bovine oviductal epithelial cells (Blondin, et al. 1997, Eyestone, et al. 1987), buffalo rat liver cells (Rehman, et al. 1994), cumulus cells (Donnay, et al. 1997, Goovaerts, et al. 2009), granulosa cells (O'Doherty, et al. 1997), endometrial cells (Le Saint, et al. 2015) and also embryonic cells (Mori, et al. 2012) improves embryo development *in vitro*. Little is known about the exact mechanisms underlying the beneficial effects of co-culture, however the secretion of paracrine embryotrophic factors is one of the putative mechanisms (for review see Orsi and Reischl 2007). These paracrine factors might be specific proteins, as oviduct specific glycoproteins (Xu, et al. 2001) or growth factors, as e.g. vascular endothelial growth factor-A (VEGF-A), VEGF-C and fibroblast growth factor (FGF-1) (Vithoulkas, et al. 2014; for review see Orsi and Reischl 2007). Furthermore, the use of a surrogate sheep oviduct *in vivo* culture system can more than double blastocyst development rates in several species, improving embryo quality to near *in vivo* standards (reviewed by Lazzari, et al. 2010). Interestingly, culturing embryos in groups also improves developmental rates and embryo quality in many mammalian species, including mice (Canseco, et al. 1992, Lane and Gardner 1992, Paria and Dey 1990, Salahuddin, et al. 1995), cattle (Donnay, et al. 1997, Goovaerts, et al. 2009, O'Doherty, et al. 1997, Wydooghe, et al. 2014), pigs (Stokes, et al. 2005), cats (Spindler and Wildt 2002), and humans (Almagor, et al. 1996, Ebner, et al. 2010, Moessner and Dodson 1995).

This tendency for improved embryo development in group culture has been ascribed to cooperative communication among preimplantation embryos (Paria and Dey 1990). Such trophic stimulation results from autocrine signalling factors produced and released by embryos which act upon the embryo itself or upon neighbouring embryos (Gopichandran and Leese 2006, O'Neill 2008b, O'Neill, et al. 2012, Stokes, et al. 2005). The presence of these autocrine embryotropins, acting as growth or survival factors, is the basis of the embryos' superior development in group culture (Figure 3). This cooperative effect was first observed in the 1990s, when it was demonstrated that increasing embryo density in murine and bovine embryos promoted blastocyst development (O'Doherty, et al. 1997, Paria and Dey 1990). In these species, an embryo density of one embryo per 2 µl of medium is thought to be optimal, balancing the accumulation of autocrine factors with that of waste products such as ammonium (Kato and Tsunoda 1994, Palasz and Thundathil 1998). Recently, embryo culture systems such as the well-of-the-well (WOW) system have been developed in which single embryos are cultured in small cavities, barely exceeding the diameter of the embryo, to create a semi-isolated microenvironment (Vajta, et al. 2000). The latter resembles the small amount of fluid that surrounds the *in vivo* embryo in the maternal tract and facilitates the development of optimal concentrations of autocrine factors (Matsuura 2014). The WOW system promotes superior embryo development and

quality in bovine (Hoelker, et al. 2010, Vajta, et al. 2000), mouse (Vajta, et al. 2008), porcine (Taka, et al. 2005), buffalo (Shah et al., 2008) and even human embryos (Vajta, et al. 2008), illustrating the importance of autocrine factors for embryonic survival and development.

The following part of chapter 1 will focus on the different mechanisms by which preimplantation embryos can secrete autocrine factors during development *in vitro*. The biochemical characteristics of the different autocrine embryotropins present during preimplantation embryonic development are described. Finally, the different pathways activated by autocrine embryotropins, which work synergistically to stimulate embryonic development, will be discussed.

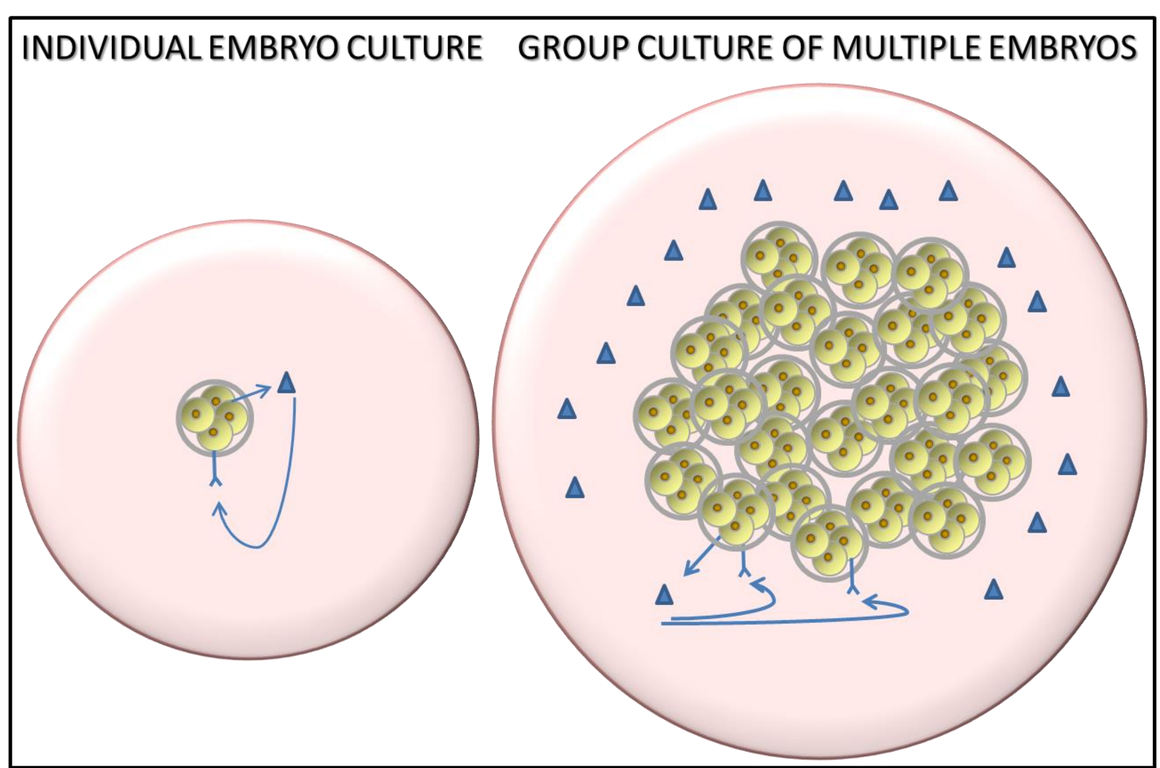


Figure 3. *Schematic illustration of the presence of autocrine embryotropins during individual culture (usually one embryo in 20 µl of medium) and group culture (often 25 embryos in 50 µl of medium).*

3.2.1 The preimplantation embryo as the origin of signalling agents

Unlike somatic cells, mammalian embryos are surrounded by an acellular zona pellucida until hatching. Subsequently, secretions can only be available for the co-cultured embryos if they can pass through the zona pellucida. This zona pellucida is composed of four glycoproteins (bZP1, bZP2, bZP3, and bZP4) and has the appearance of a sponge under electron microscopy: a complex fibrous network with many pores, the diameter and shape of which vary among species (reviewed by Van Soom, et al. 2010). In bovine embryos, the pores are >50 nm in diameter, with 20–50% >200 nm. Using fluorescently labelled markers, Legge (1995) showed that the zona pellucida of murine oocytes is

permeable to markers up to 170 kDa. Albumin, a carrier protein of several autocrine embryotropins, has a molecular weight of only 66.5 kDa and an oblong shape, 3.5 nm in length. Hence, theoretically, it can pass through the pores. Likewise, exosomes of 40–100 nm diameter should be able to pass through these pores. Besides its molecular weight and size, the hydrophilic/lipophilic character of the molecule is important: most lipids and lipid-containing molecules pass through the zona pellucida relatively easy (Turner and Horobin 1997). In the next part, we will discuss that preimplantation embryos are able to produce and secrete autocrine embryotropins by several mechanisms: active secretion, passive outflow, binding to a carrier molecule, or transport within extracellular vesicles (Figure 4).

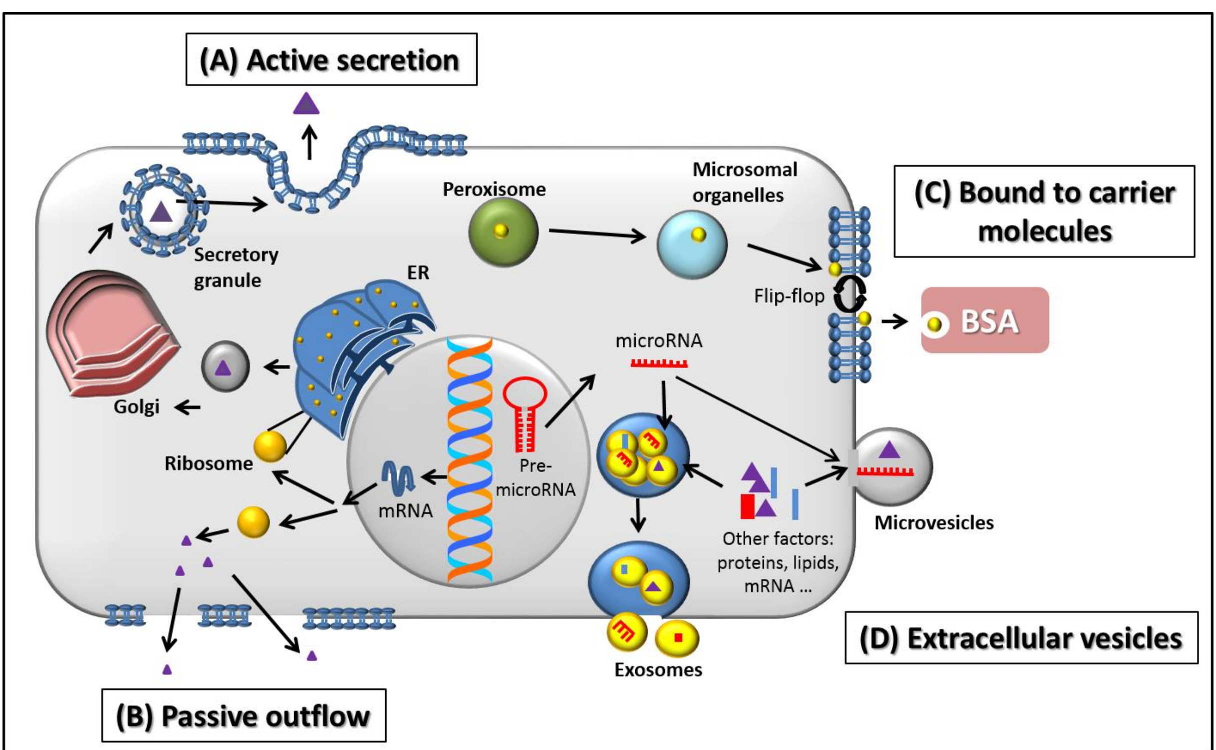


Figure 4. *Preimplantation embryos are able to produce and secrete autocrine factors by different mechanisms. (A) Active secretion. Proteins destined for secretion are generated inside ribosomes, bound to the endoplasmic reticulum (ER) and subsequently transported to the Golgi apparatus. After formation of their tertiary and quaternary structure, the proteins are transported inside secretory granules to the cell surface and released into the culture medium. (B) Hydrophilic compounds like proteins can be released passively by cells following membrane damage. Likewise, intracellular proteins, formed inside free-floating ribosomes, can be released extracellularly. (C) Hydrophobic factors, such as platelet activating factor (PAF), can be released bound to a carrier molecule. De novo synthesis of PAF occurs inside peroxisomes and microsomal organelles. Subsequently, PAF becomes bound to the inner leaflet of the plasma membrane and is made available to albumin (BSA) after flip-flop passage across the membrane. (D) Proteins, lipophilic factors, and microRNA can be secreted by extracellular vesicles. Exosomes are formed intracellularly and are released by fusion of multivesicular bodies with the plasma membrane. Microvesicles, by contrast, are shed from the cell surface.*

Active secretion

The 50-year-old consensus states that proteins are secreted using the classical secretory pathway (Figure 4): briefly, secreted proteins carry a signal peptide that targets them into the endoplasmic reticulum (ER) from where they are transported to Golgi apparatus and packaged into secretory vesicles (Palade, 1975). These vesicles convey their cargo to the cell surface, where they fuse with the plasma membrane to release their content into the extracellular space. In specialised secretory cells such as neurons, endocrine and exocrine secretory cells, and in hematopoietic cells, certain proteins are released only after the cell receives an appropriate biochemical or electrical stimulus and is therefore called regulated secretory pathway. By contrast, the constitutive secretory pathway is present in all eukaryotic cells in which certain proteins move to the cell surface in transport vesicles and are secreted continuously (Burgess and Kelly 1987; Lodish, et al. 2003).

Besides this classical secretory pathway, recently it has been shown that proteins can also be secreted without entering the ER–Golgi conventional pathway of secretion. This alternative route of secretion is called unconventional protein secretion and is largely triggered by stress. Unconventional protein secretion (UPS) is complex and includes the translocation of proteins without a signal peptide across the plasma membrane, but also the secretion of vesicles that reach the plasma membrane by bypassing the Golgi complex, despite entering the endoplasmic reticulum (ER) (for review, see Rabouille, 2017). To the best of our knowledge, no electron microscope or fluorescent-probe studies have been conducted in preimplantation embryos that could detect which of the different pathways of secretion is present in embryos.

Passive outflow

Hydrophilic molecules can also be released passively into the culture medium by blastomeres with damaged cell membranes. We have shown that the percentage of membrane-damaged cells (MDC ratio) in an average population of day 8 bovine *in vitro*-produced embryos is surprisingly high: only 12.5% of embryos showing normal development [i.e. >64 cells at 192 hours post-insemination (hpi)] had no membrane damaged cells. Interestingly, almost half of the arrested embryos (<64 cells at 192 hpi) had an MDC ratio of >50%, indicating that poor quality embryos consist predominantly of membrane-damaged cells and thus might passively release numerous proteins into the medium (Wydooghe, et al. 2011).

Bound to a carrier protein

Albumin, often the most abundant protein present in culture media, acts as a molecular carrier for hydrophobic autocrine factors produced by embryos. Hydrophobic molecules can bind to albumin at a hydrophobic core located between amino acids 240 and 386 (Ammit and O'Neill 1997a, Brodersen, et al. 1990, Brown and Shockley 1982). The best-known example of this interaction is the release of 1-o-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine or platelet activating factor (PAF) bound to albumin by murine embryos cultured *in vitro* (for review, see O'Neill 2005). After *de novo* synthesis of PAF inside peroxisomes and microsomal organelles and their transport to the plasma membrane of the blastomeres, PAF flip-flops across the membrane bilayers to be available for release. The secretion of PAF into the culture medium requires albumin as an acceptor molecule: more PAF was released by murine embryos at higher albumin concentrations (Ammit and O'Neill 1997a). A conformational change of albumin, involving cysteine–cysteine disulphide breakdown, is required for it to load PAF, since the tertiary structure of albumin contains 17 interchain disulphide bonds that link 34 of the available 35 cysteine residues thereby preventing access to the hydrophobic core (Ammit and O'Neill 1997b).

Extracellular vesicles

Besides direct secretion into the culture medium, bioactive molecules can also be secreted in spherical, bilayered lipid vesicles. These extracellular vesicles recently gained attention as mediators of intercellular communication. By local and long-distance interactions with target cells, they exert pleiotropic effects in the maintenance of normal physiology, including tissue repair, stem cell maintenance, and blood coagulation, but also in diverse pathological conditions, like cancer, neurodegeneration, and autoimmune and infectious diseases (reviewed by Yoon, et al. 2014). Extracellular vesicles are present in many, perhaps all, biological fluids as well as in the medium of cultured cells, including epithelial cells (van Niel, et al. 2001), neurons (Faure, et al. 2006), Schwann cells (Fevrier, et al. 2004), and tumour cells (Wolfers, et al. 2001). They contain a variety of bioactive molecules such as proteins (e.g. membrane, cytosolic, and cytoskeleton proteins), bioactive lipids (e.g. sphingomyelin and prostaglandins), and genetic material [e.g. mRNA, microRNAs, rRNA, and mitochondrial DNA (reviewed by Raposo and Stoorvogel 2013)]. Based on their biogenesis, extracellular vesicles can be categorized into microvesicles and exosomes. Microvesicles originate from the plasma membrane by outward budding and splitting while exosomes are formed in endosomal compartments called multivesicular bodies and are secreted upon fusion of these bodies with the plasma membrane to release their contents as exosomes (reviewed by Bobrie, et al. 2011). Exosomes

also play an important role in inter-embryonic communication, as demonstrated by Saadeldin *et al*. (2014) who showed that the addition of exosomes isolated from the conditioned medium of parthenogenetic embryos could increase the developmental competence of cloned embryos. Whether this positive influence was caused by proteins, lipids, or microRNAs present in these exosomes remains to be clarified. It is possible that proteins transported within exosomes are better protected against degradation relative to proteins secreted into the culture medium and are perhaps more accessible to co-cultured embryos. It would be interesting to add exosome-encapsulated proteins with possible autocrine functions to the culture medium of preimplantation embryos and compare these results with the addition of the protein alone. Clearly, more research is needed to determine the contents of exosomes and their possible role in inter-embryonic communication.

3.2.2 Characteristics of The Signalling Agents

Inter-embryonic communication is mediated by a wide range of biochemical messengers including proteins, lipids, neurotransmitters, saccharides, nucleotides and microRNAs.

Proteins/peptides

In contrast to somatic cells, cleaving embryos are not dependent upon RNA transcription for protein synthesis before embryonic genome activation (EGA). Both maternal mRNA and proteins that have accumulated in the cytoplasm of the growing oocyte are used by the cleaving embryo. This maternal contribution sustains and directs early development until the 1- to 2-cell stage in mice (Schultz 1993), the 4-to 8-cell stage in humans (Braude, et al. 1988), and the 8–16 cell stage in cattle (Graf, et al. 2014, Jiang, et al. 2014), and even to the 4000-cell stage in Xenopus laevis (Newport and Kirschner 1982). As a result of this transcriptional silence, protein synthesis during the first cleavages is initiated by a translational control mechanism exhibited by stored maternal mRNAs (reviewed by Kristiansson and Seli 2013). A cytoplasmic polyadenylation element binding protein (CPEBP) regulates the initiation of translation in oocytes and early embryos through polyadenylation of maternal mRNA. CPEBP has been identified in murine (Seli, et al. 2005) and human embryos (Guzeloglu-Kayisli, et al. 2008). When CPEBP is bound to a repressor protein called maskin, translation is blocked, while phosphorylation of CPEBP leads to activation of a CPEBP-associated poly(A) polymerase complex which, in turn, results in elongation of the short poly(A) tail of mRNA. This longer poly(A) tail will bind poly(A)-binding protein, which recruits the eukaryotic initiation factor 4G (eIF4G) to replace maskin thereby resulting in increased translation (reviewed by Vasudevan, et al. 2006). This changes at the time of embryonic genome activation (EGA), from that time the embryo takes charge of gene expression: maternal mRNA

are degraded and embryonic mRNA transcription begins (Li, et al. 2013). After EGA, protein synthesis is initiated in a similar manner to that in somatic cells, starting from de novo synthesis of embryonic mRNA. Consequently, the total mRNA content of embryos increases rapidly after EGA (Gilbert, et al. 2009).

Translation of mRNA into proteins requires ribosomes. During oocyte maturation an appropriate quantity of ribosomes must be stored in the cytoplasm to support protein synthesis during early cleavage. Ribosomes are mainly synthesized during the germinal vesicle stage of oocyte maturation when a functional nucleolus necessary for the transcription of ribosomal RNA (rRNA) genes is present (Ferreira, et al. 2009). Close to the time of EGA, a functional ribosome-synthesizing nucleolus develops from maternal inactive nucleolus precursor bodies (Hyttel, et al. 2000, Maddox-Hyttel, et al. 2007). As in somatic cells, the newly synthesized proteins in the ribosomes bound to the endoplasmatic reticulum (ER) are transported to the Golgi apparatus in transitional elements. Proteins destined for secretion are packaged into secretory vesicles which move to the site of secretion and are secreted via the constitutive secretory pathway (Hong and Tang 1993). Controversy exists about the functionality of the ER-Golgi secretory pathway in preimplantation embryos before EGA, since supplementation of the culture medium with Brefeldin A (BFA), a fungal metabolite which interrupts ER-to-Golgi vesicle transport, does not inhibit bovine embryos from reaching the 8-cell stage (Payne and Schatten 2003). Likewise, BFA does not inhibit cleavage to the 2-cell stage in mice (Clayton, et al. 1995). On the other hand, analysis of the culture medium of murine embryos by mass spectrometry revealed the presence of secreted proteins after only 24 h of culture (Katz-Jaffe, et al. 2006) but further research is needed to determine whether these proteins were secreted by the ER–Golgi secretory pathway.

It is has been known for some time that preimplantation embryos secrete cytokines and growth factors such as interleukin-1 (IL-1), IL-6, colony stimulating factor-1 (CSF-1), transforming growth factor (TGF), and tumour necrosis factor (TNF) (see supplementary table 1) (Watson, et al. 1992, Zolti, et al. 1991). Moreover, the presence of the corresponding receptor for each of these proteins on preimplantation embryos (see supplementary table 2) strongly suggests an autocrine role for these molecules. It has been shown that murine and human embryos secrete survivin, a member of the apoptosis-inhibiting protein family that regulates cell division and inhibits apoptosis (Balakier, et al. 2013, Kawamura, et al. 2003). Preimplantation embryos also secrete the peptide preimplantation factor (PIF) that has similar activities to cytokines although it is not classified as such due to its small size. In culture, PIF is secreted by the 2-cell stage murine embryo (Stamatkin, et al. 2011a). Addition of PIF to a culture system for single bovine embryos increased their survival under adverse conditions (Stamatkin, et al. 2011b). Recently, it was shown that PIF is assimilated by viable embryos and binds

intracellularly to proteins involved in regulating oxidative stress and protein misfolding, such as protein disulphide isomerase, peroxiredoxin 4, and heat shock proteins (Barnea, et al. 2014).

Lipids

Bioactive lipids have been shown to have critical cell signalling functions, both intra- and intercellularly (English 1996, Hannun and Obeid 2008, Spiegel and Milstien 2003). Lipids with possible autocrine actions on preimplantation embryos include phospholipids, fatty acids and eicosanoids.

Phospholipids

Phospholipids and cholesterol are the most abundant lipids in membranes. During early embryogenesis, the enrichment of phospholipids and cholesterol is crucial to form cell membranes to accommodate rapid cell division. Additionally, phospholipids have important roles in cell signalling: (i) intracellularly, as phosphatidylinositol derivatives, they play a crucial role in intracellular signalling transduction (e.g. in the phosphatidylinositol 3-kinase-dependent signalling pathway); (ii) extracellularly, as first messenger or autocrine factors, such as PAF, lysophosphatidic acid and sphingosine-1 phosphate.

PAF produced by preimplantation embryos was the first autocrine embryotropin identified (Collier, et al. 1988, O'Neill 1985). Embryos release PAF in high quantities, ranging from 1 to up to several hundreds of ng of PAF per embryo per 24 h (Ammit and O'Neill 1991, Roudebush, et al. 2002). Vandenberghe et al. (2014) showed that bovine embryos express the PAF receptor throughout development, with expression levels increasing until the 16-cell stage and then decreasing significantly in the post-compaction stages. PAF is known to increase mitosis (Roberts, et al. 1993), blastocyst cell number, and implantation rate after embryo transfer in mice (Ryan, et al. 1990) and also may act as a survival factor (O'Neill 1998). Addition of exogenous PAF to the medium increases the rate of day 8 blastocyst development in bovine embryos and results in a more energy-efficient oxidative metabolism pathway (Gopichandran and Leese 2006).

Lysophosphatidic acid (LPA) is a cell membrane phospholipid metabolite that can act as an extracellular signal to affect different aspects of reproduction by binding to a G-protein-coupled LPA receptor (reviewed by Ye and Chun 2010). Recently, mRNA transcripts and protein expression of enzymes involved in LPA synthesis and LPA receptors were identified in late-stage cleavage bovine embryos and blastocysts. LPA has been detected in bovine embryo-conditioned medium (Torres, et al. 2014). Addition of LPA to the culture medium increased blastocyst development of murine embryos

(Kobayashi, et al. 1994), but not of bovine embryos, although transcription levels of embryo quality markers were certainly affected in the latter; e.g. Bcl2-Associated X (BAX) (apoptotic) decreased while B-cell lymphoma 2 (BCL2) (anti-apoptotic) and insulin-like growth factor 2 receptor IGF2R (growth marker) increased (Torres, et al. 2014). Another bioactive cell membrane metabolite is sphingosine-1 phosphate (S1P), a potent anti-apoptotic substance that acts by binding a family of five G-protein-coupled receptors. Addition of S1P protects murine embryos against the apoptotic damage induced by ceramide (Geng, et al. 2012, Guo, et al. 2013). In addition, supplementation of porcine embryo culture medium with S1P increases blastocyst development and produces a higher total cell number and lower rate of apoptotic cells (Hyo-Sang and Deog-Bon 2012). Finally, S1P has been reported to reduce the fragmentation rate of human preimplantation embryos and, hence, to increase embryo quality (Hannoun, et al. 2010).

Fatty acids

During *in vitro* maturation of oocytes, fatty acids are incorporated into the cytoplasm (Kim, et al. 2001) where they are stored in lipid droplets as triglycerides (Aardema, et al. 2011, Abe, et al. 2002, Cran 1985, Leroy, et al. 2005a, Sturmey, et al. 2006). The most abundant intracellular fatty acids in porcine, bovine, and ovine oocytes are palmitic acid, oleic acid, and stearic acid, which account for around 70% of the fatty acids present (Homa, et al. 1986, Leroy, et al. 2005b, McEvoy, et al. 2000). They are used mainly in mitochondrial β-oxidation to generate ATP during oocyte maturation and early embryonic development (Dunning, et al. 2010, Sturmey and Leese 2003). Quinn and Whittingham (1982) demonstrated that addition of exogenous oleic and palmitic acids inhibited fertilisation of murine oocytes but increased blastocyst formation and hatching rate when supplemented to 8-cell embryos. Subsequently, a negative impact on embryo development was observed when oocytes and early embryos were exposed to high concentrations of palmitic and stearic acids (Leroy, et al. 2005b, Van Hoeck, et al. 2012, Van Hoeck, et al. 2011).

In addition to functioning as an endogenous energy substrate, fatty acids take part in cell-to-cell communication and can be transported bound to carrier proteins such as serum albumin, lipoproteins, or fatty acid binding proteins. Exosomes of a basophil cell line can carry a large range of free fatty acids, including both saturated (e.g. palmitic and stearic acids) and unsaturated (e.g. arachidonic acid), in addition to the enzymes involved in lipid metabolism , such as phospholipase (PLA) (Record, et al. 2014, Subra, et al. 2010). Whether the exosomes released by preimplantation embryos contain fatty acids and their enzymes remains to be elucidated.

Eicosanoids

Synthesis of eicosanoids from membrane phospholipids requires two key steps: (i) activation of phospholipase (PLA) to bring about the release of free arachidonic acid; and (ii) oxidation of arachidonic acid by two families of enzymes, lipoxygenase (LOX) to produce leukotrienes and cyclo-oxygenase (COX-1 & -2) which generates prostaglandins (PGs), prostacyclins (PGIs), and thromboxanes. Of these molecules, PGs have been the best studied during the preimplantation period. Production of PGs has been confirmed for bovine, ovine, and porcine blastocysts (reviewed by Lewis 1989), and for day 10–32 equine embryos (Stout and Allen 2002, Watson and Sertich 1989). Western blot analysis has shown that COX-1, COX-2, and prostacyclin synthase (PGIS) proteins are expressed by murine blastocysts (Huang, et al. 2004). In human embryos, mRNA expression of PLA2 and COX has been demonstrated throughout the preimplantation period (Wang, et al. 2002), thereby confirming the results of an earlier study in which PGE2 was detected in the culture medium used for human embryos (Holmes, et al. 1990).

Embryo-derived PGE increases ion/water transport across tight junctions between the inner cell mass and the trophectoderm and has an important role in blastocyst expansion and hatching of murine embryos (Baskar, et al. 1981). More recently, analysis of eicosanoids by reverse-phase high-performance liquid chromatography (HPLC) revealed that murine embryos synthesize predominantly prostacyclin (PGI2) from exogenous arachidonic acid (Huang, et al. 2004, Pakrasi and Jain 2007). Adding a COX-2 inhibitor significantly decreased blastocyst development and hatching rate, whereas addition of a PGI2 analogue improved blastocyst development by increasing total cell number (Pakrasi and Jain 2007) and hatching rate of murine embryos (Huang, et al. 2004, Liu, et al. 2006). In bovine embryos *in vitro,* supplementation of the culture medium with a PGI2 analogue improves embryo kinetics by increasing the proportion of expanded blastocysts, and embryo quality by decreasing the number of apoptotic cells (Song, et al. 2009).

MicroRNAs

MicroRNAs are a family of short, non-coding RNAs (17–25 nucleotides) with an important role in gene regulation. They are able to inhibit translation by binding of the seed region(s) to the microRNA binding region of their target mRNA, thereby promoting their own degradation in the cytoplasm, or by blocking the translation machinery (He and Hannon 2004). MicroRNAs also play an important role during preimplantation development. Studies in mice have shown significant inheritance of maternal microRNAs in the zygote to be crucial for the early stages of embryonic development (Tang, et al. 2007). Furthermore, microRNA levels undergo dynamic changes during preimplantation development

of murine embryos (Mineno, et al. 2006, Viswanathan, et al. 2009, Yang, et al. 2008): for example, total microRNA in 4-cell stage embryos is double that at the 2-cell stage (Tang, et al. 2007). In bovine embryos, Goossens et al. (2013) showed that microRNAs regulate the balance between pluripotency and differentiation into trophectoderm and inner cell mass. Besides their known function in gene regulation, secreted microRNAs have recently received increasing attention for their role in intercellular communication and the manner in which secreted microRNAs from donor cells influence gene expression of recipient cells (Valadi, et al. 2007). MicroRNAs must be transported by exosomes to protect them from RNAse activity. Exosomes can be internalized by target cells via endocytosis, phagocytosis, or direct fusion with the plasma membrane; whether these actions are receptor-mediated remains to be elucidated (Chen, et al. 2012). Numerous microRNAs can be incorporated simultaneously to affect the translation of multiple mRNAs in the target cell. This means of communication is important *in vivo* for pregnancy recognition between the viable blastocyst and the receptive endometrium (reviewed by Galliano and Pellicer 2014). It has been shown that human, bovine (Kropp, et al. 2014), and porcine (Saadeldin, et al. 2014) embryos secrete exosomes into the surrounding medium during preimplantation development *in vitro*. In human and bovine embryos, differential expression of microRNA in exosomes isolated from culture medium was observed between degenerate embryos and those that reached the blastocyst stage (Kropp, et al. 2014). Furthermore, microRNAs detected in culture medium from human embryos are expressed differentially according to the method of fertilisation [*in vitro* fertilisation (IVF) versus intracytoplasmic sperm injection (ICSI)], chromosomal status (e.g. aneuploidy), and pregnancy outcome (Rosenbluth, et al. 2014).

Other molecules

Hyaluronic acid

Hyaluronic acid (HA), a high molecular weight polysaccharide, is one of the most abundant glycosaminoglycans in uterine, oviductal, and follicular fluids in the mouse (Sato, et al. 1987), pig (Archibong, et al. 1989), human (Suchanek, et al. 1994), and cow (Lee and Ax 1984). *In vitro*, bovine preimplantation embryos secrete HA into the culture medium (Marei, et al. 2013, Stojkovic, et al. 2003) and inhibition of HA synthesis by a specific inhibitor results in a decrease in blastocyst formation (Marei, et al. 2013). Addition of HA to the culture medium, stimulates *in vitro* development of bovine (Furnus, et al. 1998, Stojkovic, et al. 2002), porcine (Miyano, et al. 1994, Toyokawa, et al. 2005), and mouse (Gardner, et al. 1999) embryos. The principal cell membrane receptor for HA, CD44, is present in 1- to 8-cell human embryos (Campbell, et al. 1995) and throughout preimplantation development in bovine embryos (Furnus, et al. 2003). Very low molecular weight HA fragments (50–60 saccharides)

mediate its action through the CD44 receptor. Recently, Marei et al. (2013) showed that bovine compacted morulas and blastocysts express hyaluronidase-2 (HYAL2), an enzyme necessary to degrade HA into these low molecular weight fragments. Binding of these smaller HA fragments to CD44 can modulate cell division and apoptosis by activating the mitogen-activated protein kinase (MAPK) pathway (see Section 3.2.3) (Marei, et al. 2013).

Nucleotides

Nucleotides serve as the building blocks of nucleic acids and in the form of nucleoside triphosphates are important carriers of energy in all organisms. Purinergic signalling is now considered to be a general intercellular communication system of many, if not all, tissues (Burnstock 1997, 2014). There are three distinct classes of purinergic receptor, known as P1, P2X, and P2Y. P1 and P2Y are G-coupled protein receptors, whereas P2X receptors are ligand-gated ion channels, transmembrane ion channels which open to allow ions to pass in response to binding of P2X. To date, only the latter are known to be present in murine embryos during early cleavage (Masse and Dale 2012). Extracellular ATP activates the P2X receptor, whereas extracellular hydrolysis by cell surface-located ectonucleotidases is necessary to activate P2Y (by ADP and adenosine) and P1 (by adenosine). In murine embryos, the ectonucleotidases embryonic alkaline phosphatase and tissue non-specific alkaline phosphatase are present during early cleavage (Masse and Dale 2012). Unfortunately, no further information is available about the role of extracellular ATP or other nucleoside triphosphates during early mammalian embryonic development. However, it has been shown that addition of nucleotides such as adenosine, inosine, and hypoxanthine reversibly blocks development of 2-cell embryos of some strains of mice (Nureddin, et al. 1990).

Biogenic amines

Biogenic amines can play an important role in embryogenesis (reviewed by Cikos, et al. 2011). Well-studied biogenic amines in relation to reproduction include catecholamines, histamine, and serotonin, and it has been shown that the rate-limiting enzyme for serotonin production, tryptophan hydroxylase-2 (TPH2), is expressed in murine oocytes and 2-cell embryos (Basu, et al. 2008). However, it is not yet known whether serotonin is actually secreted by preimplantation embryos. Serotonin can bind to different forms of G-coupled protein receptors that show variation in expression during preimplantation development (Amireault and Dube 2005, Cikos, et al. 2007, Cikos, et al. 2005, Il'kova, et al. 2004, Vesela, et al. 2003). It has been suggested that serotonin can activate different signalling pathways at different developmental stages, including the MAPK pathway (reviewed by Cikos, et al.

2011). Addition of serotonin to *in vitro* cultured murine embryos significantly reduced total cell number and increased the incidence of apoptotic cells (Il'kova, et al. 2004, Vesela, et al. 2003). This contrasts with the *in vivo* situation where decreasing maternal serotonin levels in pregnant mice by administering an inhibitor p-chlorophenylalanine of TPH2, impaired development of preimplantation embryos by causing a reduction in cell proliferation and inducing abnormalities in blastocyst formation (Khozhai, et al. 1995). Clearly, the effects of serotonin on preimplantation embryo development are context dependent.

3.2.3 Signalling Agents Act Through Different Pathways

The autocrine signalling factors secreted by preimplantation embryos might have a survival function by activating the phosphatidylinositol-4,5,-bisphosphate 3-kinase (PI3K) pathway. They may also act as growth factors by activating the MAPK pathway. Finally, by their activation of the peroxisome-proliferator-activated receptor family (PPAR) pathway, lipophilic autocrine embryotropins serve both survival and anti-apoptotic functions (Figure 5).

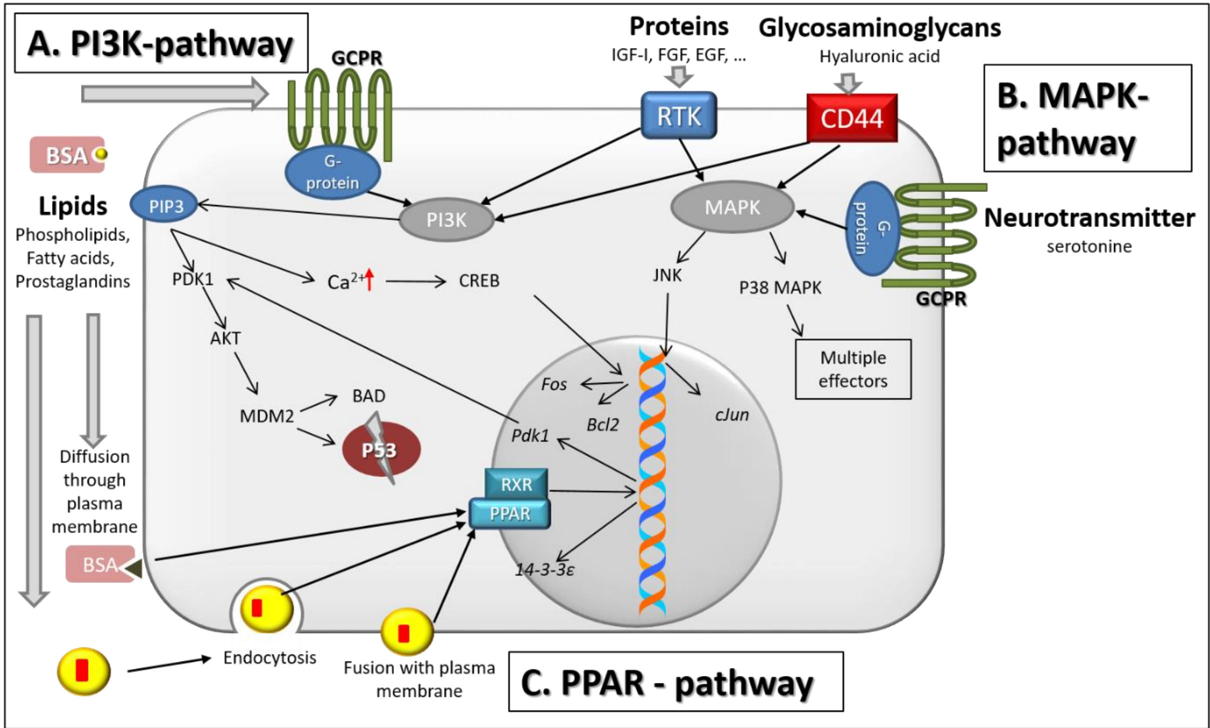


Figure 5. *Overview of the different pathways activated by autocrine embryotropins. (A) The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway can be activated by the binding of (i) proteins to a tyrosine kinase receptor (RTK), (ii) hyaluronic acid to CD44, or (iii) phospholipids to their G-coupled protein receptor (GCPR). This will result in the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which might activate two different pathways. The AKT-dependent pathway requires the activation of 3-phosphoinositide-dependent protein kinase 1 (PDK1), which in turn, will*

activate AKT, a serine threonine kinase that mediates its function by activating the murine double minute 2 (MDM2) protein. Subsequently, this leads to degradation and inhibition of the proapoptotic mediators, P53 and Bcl2-associated death promoter (BAD). Alternatively, the PI3K pathway can result in Ca^{2+} transients activating cAMP response element-binding protein (CREB), a transcription factor, to initiate the transcription of Fos, important for cell proliferation, and Bcl2, which plays a role in inhibiting the proapoptotic protein, Bcl2-Associated X (BAX). (B) The mitogen-activated protein kinase (MAPK) pathway can be activated by proteins, hyaluronic acid, or serotonin binding to their corresponding RTK, CD44, or GCPR, respectively. This activates the c-Jun N-terminal kinase (JNK) pathway and the p38 MAPK pathway to result in a pro-mitogenic signal. (C) Lipophilic autocrine embryotropins such as fatty acids or prostaglandins are able to diffuse through the plasma membrane, or can be internalized by endocytosis or fusion of exosomes. Subsequent binding to a nuclear receptor of the peroxisome-proliferator activated receptor family (PPAR) will up-regulate transcription of PDK-1, protecting cells from apoptosis, and 14-3-3ε´ involved in cell-cycle checkpoints. Abbreviations: BSA, bovine serum albumin; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF-I ,insulin-like growth factor-I ; RXR, retinoid X receptor.

PI3K pathway

The PI3K pathway is a survival pathway activated by the binding of (i) protein growth factors such as insulin-like growth factor I (IGF-I) to their tyrosine kinase receptors (RTKs) such as IGF-IR (Navarrete Santos, et al. 2004), (ii) bioactive lipids like PAF to a G-coupled protein receptor (GCPR) such as PAF-R (O'Neill 2005), or (iii) hyaluronic acid to its CD44 receptor (Toole 2001). Binding of an autocrine embryotropin to its receptor triggers a canonical signalling pathway initiated by the activation of PI3K. This leads to the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) which, in turn, triggers two different signalling pathways in preimplantation embryos, an AKT-dependent pathway and the PI3K-dependent calcium signalling pathway (reviewed by O'Neill, et al. 2012).

AKT-dependent signalling pathway

An important function of PIP3 is to allow the recruitment of kinases, such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) and their substrates to facilitate phosphorylation. The primary target of PDK1 is AKT, a serine threonine kinase that mediates its action by activating the murine double minute 2 (MDM2) protein (Franke, et al. 1997). Subsequently, the pro-apoptotic mediators, P53 and Bcl2-associated death promoter (BAD), are inhibited and degraded, meaning that this pathway is involved in the survival of the preimplantation embryo (reviewed by O'Neill 2008a). This AKT-dependent pathway is present in murine embryos from the 1-cell stage through to the blastocyst stage (Riley, et al. 2005). Navarrete Santos et al. (2008) showed that after

the first lineage segregation in rabbit blastocysts, the PI3K/ATK-dependent pathway is present in both inner cell mass and trophectoderm.

PI3K-dependent calcium signalling pathway

The PI3K-dependent calcium signalling pathway has been studied in murine preimplantation embryos (O'Neill, et al. 2012). Binding of PAF to its G protein-coupled receptor results in Ca2+ transients caused by the release of intracellular calcium stores and an influx of external calcium (Emerson, et al. 2000, Roudebush, et al. 1997). The Ca2+ transients activate cAMP response element-binding protein (CREB), a transcription factor, leading to the transcription of, among others, Fos and Bcl2. Fos is involved in cell proliferation, differentiation, and survival, while Bcl2 plays a role in the inhibition of the pro-apoptotic protein, BAX.

MAPK pathway

MAPK pathways regulate the proliferation, growth, differentiation, and death of cells (reviewed by Ono and Han 2000) and are present throughout preimplantation development in mice (Wang, et al. 2004). Activation of a tyrosine kinase receptor by the binding of a protein such as an autocrine embryotropin or activation of CD44 by the binding of a glycosaminoglycan such as hyaluronic acid leads to the initiation of the MAPK cascades. Binding of serotonin to its GCPR also activates this pathway. MAPK pathways include the c-Jun N-terminal kinase (JNK) pathway and the p38 MAPK pathway, essential for normal preimplantation development of murine embryos (Maekawa, et al. 2005). JNK will activate the transcription factor, cJun, which is required for progression through the G1 phase of the cell cycle. Activated p38 MAPK functions through multiple effectors, including several transcription factors as well as protein kinases. Targeted inactivation of the p38 MAPK by specific inhibitors induces developmental arrest of murine embryos at the 8- to 16-cell stage (Natale, et al. 2004).

PPAR pathway

Lipophilic autocrine embryotropins, for example PGs or fatty acids, are transported by binding to transport proteins present in the culture medium or inside exosomes. They are able to pass through the cell membrane and bind to a nuclear receptor PPAR, which consists of α, β/δ, and γ isoforms. PPARs form heterodimers with another type of nuclear receptor, retinoid X receptor (RXR), and activation in this way leads to the expression of genes involved mainly in lipid and carbohydrate metabolism (Desvergne and Wahli 1999, Kliewer, et al. 1997). Activation of the PPARδ nuclear receptor

by prostaglandin I2 upregulates gene transcription of PDK-1, involved in protecting cells from apoptosis, and of 14-3-3ε´, involved in cell-cycle checkpoints (Di-Poi, et al. 2002). Transcripts of PPAR-γ decrease in concentration in bovine preimplantation embryos between the 2-cell and morula stages, with a renewed upsurge at the blastocyst stage. Transcripts for RXRα and RXRβ were detected from the 2-cell stage through to the hatched blastocyst (Mohan, et al. 2002).

3.3 Individual *in vitro* bovine embryo culture

Bovine embryos cultured in group thrive better than individually cultured embryos in terms of embryo development and embryo quality (Donnay, et al. 1997, Goovaerts, et al. 2009, O'Doherty, et al. 1997). Therefore, in bovine scientific IVP protocols, embryos are mostly grouped together irrespective of their origin, in order to obtain large groups of 25 embryos in a drop of 50 µl medium. In contrast, in human IVF, standard group culture cannot be adopted for two reasons. First, human oocytes from different donors can obviously not be combined since each oocyte must be traceable back to the original donor, and second, human embryo quality assessment requires an individual follow-up of each embryo during the entire culture period to be able to select the best embryo for transfer. By contrast, the development of an individual *in vitro* bovine embryo culture system has been a challenge, however it would be a valuable tool to increase the knowledge on folliculogenesis, oogenesis, embryo metabolism. The obtained knowledge could improve bovine commercial IPV systems, where culture in very small groups is often required but could also be extrapolated to improve human IVP systems. Furthermore, the development of an individual oocyte culture system is a valuable tool for toxicity screening purposes (for review see Goovaerts, et al. 2010, Bols, et al. 2012). As discussed earlier, individual culture of embryos usually impairs embryo development because of the lack of autocrine factors. However, to counteract these low levels of growth factors several different media supplementations have been tested to support individual embryo development, or alternatively co-culture with somatic cells or the use of conditioned medium to supply sufficient growth factors to the individually cultured embryos was tested (for review see Bols, et al. 2012). Importantly besides the different media formulations or co-culture sytems, when looking at studies investigating individual embryo culture striking differences can be observed. Firstly, studies might differ in the way oocytes are matured and fertilised: this can be performed in groups (G-G) and subsequently the embryos are cultured individually (I) (G-G-I) or oocytes can be handled individually from the start of the IVP procedure (I-I-I). Furthermore, during culture period embryos can be cultured using 2 different approaches: i) a complete individual culture system, meaning that each embryo is cultured in a separate drop of culture medium separately covered by mineral oil or ii) according to the ‘apart together strategy’, meaning that each embryo is cultured in a separate drop of culture medium,

however several drops in the same dish are covered together by mineral oil. Likewise, the exchange of lipophilic autocrine factors in the apart together strategy cannot be excluded (Figure 6).

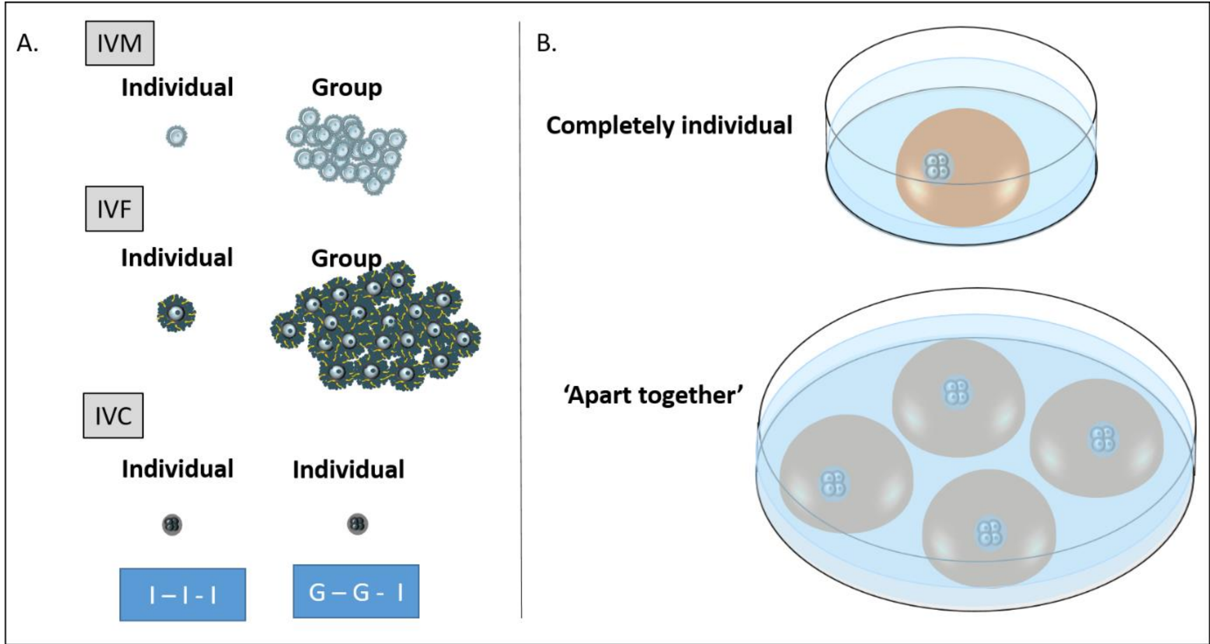


Figure 6: *Different approaches for individual in vitro bovine embryo culture. A. Oocytes might be handled individually during the whole IVP process: in vitro maturation, fertilisation and culture (I-I-I), or they can be matured and fertilised in groups (of different size depending on the study) and subsequently cultured individually (G-G-I). B. During in vitro culture, embryos can be cultured completely individual: in separate culture drops which are covered singly by mineral oil (no exchange of embryotrophic factors possible). In the 'apart together' strategy, embryos are cultured in separate culture drops, covered together by mineral oil. In this system, lipophilic embryotrophic factors can be exchanged.*

4. Bovine group culture system as a model

Although *in vitro* production of human embryos is daily practice nowadays as a treatment for infertile couples, it is still important to increase the understanding of embryo biology. In this thesis, we want to increase the knowledge of the bovine group culture system, and use this system as a model to enhance human IVF procedures. Up till now, the most popular animal model for preimplantation embryo development is the mouse, popular because of its availability, small size, relatively low cost, ease of handling, and fast reproduction rate. A unique advantage of the mouse model is the availability of inbred strains, nearly genetically identical and the availability of tools to create genetically modified mice that provide an opportunity for studying gene function (Taft 2008). However, the widespread use of mouse embryos as a model for preimplantation embryo development is more likely due to the advantages of the mouse as a model rather than the characteristics of the mouse embryo.

Recently bovine embryos have been put forward as a valuable alternative model for preimplantation embryo development (Hansen 2010, Menezo and Herubel 2002, Santos, et al. 2014, Van Soom, et al. 2011). In certain important aspects, cows reflect far more closely the situation in humans than mice. Firstly, the bovine genome is more similar to the human genome than the mouse genome. Consequently, the amino acid sequence of most proteins is more conserved between cattle and humans than between mice and humans (reviewed by Hansen 2010). As a result, the proteins involved with fertilisation or embryonic development of human oocytes are more closely related to bovine oocytes than mouse oocytes (Sylvestre, et al. 2013). Transcriptome analysis revealed that mRNA profiles of human preimplantation embryos share more similarity to those of the bovine than those of the mouse (Jiang, et al. 2014).

Furthermore, a bovine model shows more similarities concerning reproductive physiology compared with humans than mice: i) mice are polytocous, producing a litter of 1 – 10 pups at a time, in contrast to cows and human, which are monotocous species producing single offspring at birth; ii) mice have a very short oestrous cycle of only 4-6 days, whereas the frequency of the oestrous cycle of cows (21 days) is more similar to that of humans (28 days) and iii) the duration of gestation is also much shorter in mice (~ 19-21 days) compared to human (~ 280 days) and bovine (~ 280 days).

Additionally, bovine embryonic development shows more similarities compared to human embryonic development than mouse embryos. For example the oocyte diameter, the time period of oocyte maturation and initial embryo development as the time to reach the 2-cell stage, morula to blastocyst transition, hatching, and the time of embryonic genome activation is more similar when human and bovine are compared (reviewed by Santos, et al. 2014). Moreover, bovine embryos are, similar to human embryos, more sensitive to adverse culture conditions than mouse embryos (Vajta, et al. 2010). Mouse embryos have varying sensitivities to deleterious effects depending on the strain (Dandekar and Glass 1987, Dubin, et al. 1995, Scott, et al. 1993). Some strains can reach the blastocyst stage when cultured in media composed of modified tap water lacking any protein supplement (Silverman, et al. 1987). These findings raise questions regarding the reliability of mouse embryos as a model to optimize culture media for human embryos (Chronopoulou and Harper 2015). To this end, we strongly believe that bovine embryos represent a valuable alternative to optimize and test the safety and suitability of culture media for human IVF.

Finally, an important advantage of a bovine model is that it reduces the large number of laboratory animals necessary for reproductive research, since oocytes can be obtained from slaughterhouse ovaries, which are leftover organs. The research material can be obtained from healthy

animals that entered the food chain and no extra facilities are necessary to house animals (Santos, et al. 2014).

Dissimilarities between bovine and human IVF

Although bovine preimplantation embryos represent an attractive model for human IVF, there are also some dissimilarities between the human and bovine IVF process. The main difference is the aim of human IVF being a treatment for infertile couples (Boerjan, et al. 2000), whereas bovine IVF is a method for rapid genetic improvement in livestock (commercial bovine IVF) and to gain more knowledge in basic biological processes (scientific labs) (Galli, et al. 2003). Further on, we will focus on the differences between the standard protocols in human IVF labs and research bovine IVF labs.

In vitro maturation

The development of a female gamete starts during foetal life, so that at birth the ovaries contain a number of oocytes arrested in the first meiotic prophase until start of puberty. The follicular environment is responsible for preventing premature meiotic resumption until the moment of ovulation (Coticchio, et al. 2012). The *in vivo* situation is very similar in cattle and human, since in both species each cycle a single oocyte is ovulated and has finished the first meiotic division leading to the protrusion of the first polar body. However, for *in vitro* embryo production, maturation of oocytes is different in the protocols used standardly for cattle and human IVP. For the production of bovine embryos in scientific labs, where typically large numbers of embryos are produced at a time, immature oocytes are recovered from antral follicles of ovaries collected at the slaughterhouse. By isolating bovine cumulus cell-oocyte complexes (COCs) from their follicles and culturing them in maturation medium, as described earlier (part 3.1), meiotic division will resume spontaneously. After 22 hours, 80 to 90% of the oocytes reach metaphase II stage meaning that they are nuclear mature. Whereas in human IVP, in the standard protocol *in vivo* matured oocytes are collected from pre-ovulatory follicles by means of transvaginal ultrasound-guided oocyte pick-up. In order to obtain more than one *in vivo* matured oocyte per cycle, women are stimulated hormonally. Likewise, on average 10 *in vivo* matured oocytes can be retrieved at the oocyte pick-up (OPU) session. However, in recent years, the importance of IVM of human immature oocytes has increased as a treatment for patients at risk for ovarian hyper stimulation syndrome (OHSS) or in terms of fertility preservation (FP) in cancer patients. Nonetheless, up till now IVM is not part of the standard procedure in infertility clinics worldwide, because the maturation rate, evaluated by the ratio of oocytes reaching meiosis II (MII), is rather low: 30-60% in FP and 50-80% for the patients with OHSS (For review see, Hiromitsu and Yukihiro 2017).

Morphologically, oocytes from cow and human are similar in size (oocyte diameter: ± 120 µm) (Fair, et al. 1995, Griffin, et al. 2006, Otoi, et al. 1997) but human oocytes are translucent, while oocytes from cows have a dark cytoplasm, due to lipid accumulation (Sturmey, et al. 2009). Due to this dark cytoplasm, it is not possible to check fertilisation rate by means of visualisation of the 2 pronuclei with light microscopy, which is an important parameter in human IVF clinics.

In vitro fertilisation

In scientific labs, bovine oocytes are fertilized by means of *in vitro* fertilisation using frozen thawed semen of a bull, previously tested in order to obtain good results. Mostly, the same bull is used to complete all related experiments in order to standardize as much as possible. During IVF, a large group of bovine oocytes is co-incubated with purified semen as described earlier, resulting in a fertilisation rate around 70%. In contrast, human oocytes are fertilized by sperm of the partner or a donor, which can be fresh or frozen semen. As a consequence, there is a high variability in the quality and fertilisation capacity of the obtained semen. Besides conventional IVF, in which a specific amount of prepared semen is co-incubated with each oocyte in a separate drop, some cases require ICSI or direct injection of a sperm cell into the cytoplasm.

In vitro culture

Besides the group size, which is the main focus of this thesis, also media formulations used for bovine or human IVC are different: bovine embryos are mostly cultured in homemade one-step media up to 168 or 192 hpi. In commercial settings bovine embryos are usually transferred to recipient cows at 168 hpi. In human IVC two different culture systems are commercially available: the ‘back-to-nature’ or 2 step media formulations, and ‘let-the-embryo-choose’ or one-step media. Two-step media consist of a cleavage medium which supports embryo development up to 68 hpi, afterwards human normal cleaved embryos will be transferred to a blastocyst medium up to 116 hpi. They are considered to mimic the changing needs of the embryo during preimplantation development. One-step formulations are applied throughout the entire preimplantation period, without the need for a media change since all substances necessary to early embryological development are provided, and the embryo itself will adapt and utilize whatever it needs (for review see Gruber and Klein 2011). Human embryos are transferred, according to the policy of the lab, as cleaved embryos (44 or 68 hpi) or at the blastocyst stage (116 hpi).

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Supplementary Table 1. Overview of the main cytokines and growth factors secreted by preimplantation embryos and reproductive tract in the human (H), bovine (B) and mouse (M) and the methodology of identification (□ mRNA expression detected; ▪ Protein detected; ■ Secreted protein detected).

Signalling factor	PREIMPLANTATION EMBRYO						REPRODUCTIVE TRACT	
	1-cell	2-cell	4-cell	8-cell	Morula	Blastocyst	Oviduct	Uterus
CSF-1	H ■ [1] -	H ▪■ [1, 2] -	H ▪ ■ [1, 2] -	H ■ [1] -	- -	H □ [2] -	- M □ [3]	H ■ [4] M □ [3]
EGF	H ▪ [5] M ▪ [6]	H ▪ [2, 5] -	H ▪ [2, 5] -	H □▪ [5] -	H ▪ [5] M ▪ [6]	H □ ▪ [2, 5] M ▪ [6]	H □ ▪ [7-9] -	H □ ▪ [10-12] -
FGF-4	-	M □ [13]	M □ [13]	M □ [13]	M □ [13]	M □ ▪ [2, 13]	-	-
GM-CSF	-	H ▪ [2]	H ▪ [2]	-	-	H □ [2]	H □ ■ [14]	H □▪■ [15, 16]
IFN-α	-	M □ [17]	-	-	M □ [17]	-	-	-
IFN-1	-	-	-	-	-	M ▪ [18]	-	-
IGF-1	- B □ [19] -	H ▪ [2] B □ [19] M □ [20]	H ▪ [2] B □ [19] M □ [20]	H ▪ [21] B □ [19] M □ [20]	- B □ [19] M □ [20]	H □ [2] B □ [19] M □ [20]	H ▪ [22] B □ [23, 24] M □ [25]	H ▪ [22] B □ [26, 27] M □ [28]
IGF-2	H □ [29] B □ [19, 30] -	H □ [29] B □ [19, 30] M □ [31, 32]	H □ [29] B □ [19, 30] M □ [31]	H □ [29] B □ [19, 30] M □ [31]	H □ ■ [29, 33] B □ [19, 30] M □ [31]	H □ ■ [29, 33] B □ ■ [19, 30, 34] M □▪ [31, 32]	- B □ [19, 24] -	H □ [35, 36] B □ [37] -
IL-1	H ▪ [38]	H ▪ ■ [1, 38]	H ▪ ■ [1, 38]	H ▪ ■ [1, 38]	H ▪ [38]	H ▪ [38]	-	H ▪ [41]

	- -	- -	- M □ [39]	- M □ [39]	- M □ [39]	B □ ■ [40] M □ [39]	- -	B □ ■ [40] -
IL-6	H ■ [1] -	H ■ [1] -	H ■ [1] -	H ■ [1] -	- -	H □ [42] M □ ■ [43]	- -	H ▪ [41] M ▪ [44]
LIF	-	-	-	-	-	M □ ■ [43]	-	M □ [45, 46]
PDGF-A	- B □ [19] -	- B □ [19] M □ [47]	- B □ [19] M □ ▪ [48]	H □ [49] B □ [19] M □ ▪ [48]	H □ [49] B □ [19] M □ ▪ [48]	H □ [49, 50] B □ [19] M □ ▪ [47, 48]	- B □ [19] -	H ■ [51] - -
PDGF-B	-	-	-	-	-	H ■ [52]	-	H □■ [51, 53]
SCF	- -	H □ [42] -	H □ [42] -	H □ [42] -	H □ [42] -	- M □ ▪ [54]	- -	H ▪ [55] M □ [54]
TGF-α	H ▪ [5] B □ [19] M ▪ [6]	H ▪ [5] B □ [19] M □ [56]	H □ ▪ [21] B □ [19] M □ [56]	H □ ▪ [21] B □ [19] M □ [56]	H ▪ ■ [5, 33] B □ [19] M □ ▪ [6, 56]	H □ ▪ ■ [5, 33] B □ [19] M □ ▪ [6, 48, 56]	H □ ▪ [7, 8] B □ [19] M □ ▪ [25]	H □ ▪ [10, 12] - M ▪ [57]
TGF-β	B □ [19] M ▪ [58]	B □ [19] -	B □ [19] M ▪ [58, 59]	B □ [19] M□ ▪ [56, 58, 59]	B □ [19] M ▪ [56, 58, 59]	B □ [19] M □ ▪ [48, 56, 58, 59]	B □ [19] M □ ▪ [25]	- M ▪ [60]
TNF-α	-	-	H □ [42]	H ■ [1]	H □ [42]	-	H ▪ [61]	H ▪ [61]
VEGF	H □ [62] -	H □ [62] -	H □ ▪ [62] -	H □▪ [62, 63] M □ [64]	H □▪ [62, 63] M □ [64]	H □▪ [62, 63] M □ [64]	H□ ▪ [65, 66] [67] -	H □ ▪ [11, 51, 68] -

Supplementary Table 2. Overview of the receptors for the main cytokines and growth factors present on preimplantation embryos and the reproductive tract in the human (H), bovine (B) and mouse (M) and the methodology of identification (□ mRNA of the receptors detected; ■ Receptor detected by immunohistochemistry).

Receptors	PREIMPLANTATION EMBRYOS						REPRODUCTIVE TRACT	
	1-cell	2-cell	4-cell	8-cell	Morula	Blastocyst	Oviduct	Uterus
CSF-R / C-fms	H □ [42]	H □ ■ [2, 42]	H □ ■ [2, 42]	H□ ■ [2, 42]	-	H □ [2, 42]	-	H □ [70]
	-	M □ [69]	M □ [69]	M □ [69]	M □ [69]	M □ [69]	-	M □ [69]
EGF-R	H ■ [5]	H ■ [2, 5, 71]	H ■ [2, 5, 21, 71]	H ■ [2, 5, 21, 71]	-	H □ ■ [2, 5]	H □ ■ [8]	H □ ■ [11, 12, 74, 75]
	-	M □ ■ [6, 72]	M □ ■ [72]	M □ ■ [72]	M □ ■ [6, 72]	M □ ■ [6, 72, 73]	-	-
FGFR-1	-	-	-	-	-	M □ [76, 77]	-	M □ [76]
FGFR-2	B □ [19]	B □ [19]	B □ [19]	B □ [19]	-	-	B □ [19]	-
	-	-	-	-	-	M □ [77]	-	M ■ [78]
GM-CSF R	-	H □ ■ [2, 80]	H □ ■ [2, 80]	H □ ■ [2, 80]	H □ [80]	H □ [2, 80]	-	H ■ [16]
	M □ [79]	M □ [79]	-	M □ [79]	-	M □ [79]	-	M ■ [16]
IGF-I R	H □ [29]	H □ ■ [2, 29]	H □ ■ [2, 29]	H □ ■ [2, 29]	H □ [29]	H □ [2, 29]	H ■ [22]	-
	B □ [19, 30]	B □ ■ [19, 30, 81]	B □ ■ [19, 30, 81]	B □ ■ [19, 30, 81]	B □ ■ [19, 30, 81]	B □ ■ [19, 30, 81]	B □ [19, 24]	B □ [27]
	-	-	-	M □ [31]	M □ [31]	M □ [31]	-	M □■ [28, 82]
IGF-II R	H □ [29]	H □ [29]	H □ [29]	H □ [29]	H □ [29]	H □ [29]	-	-
	B □ [19, 30]	B □ [19, 30]	B □ [19, 30]	B □ [19, 30]	B □ [19, 30]	B □ [19, 30]	-	-

	-	M □ [31]	M □ [31]	M □ [31]	M□ [31]	M□ [31]	-	-
LIF-R	H □ [83] -	H □ [83] -	H □ [83] -	H □ [83] -	- -	H □ [42, 83, 84, 85] -	H □ ■ [85] -	H □ ■ [84, 85, 86] M □ [46]
PDGF-AR	- B □ [19] M □ [47]	- B □ [19] -	H □ [49] B □ [19] -	H □ [49] B □ [19] -	- B □ [19] -	H □ [49] B □ [19] M □ [47]	- B □ [19] -	H □ [50] B □ [87] -
PDGF-BR	- -	- -	- -	H □ [49] -	H □ [49] -	H □ [49] -	- -	- B □ [87]
SCFR/c-KIT protein	- -	H □ [42] -	H □ [42] -	H □ [42] -	- -	H □ [42] M □ [54]	- -	- -
TGF-β R	-	-	-	-	-	-	-	M □ [88]
TNF R	- -	H □ [42] -	H □ [42] -	H □ [42] -	- -	- M ■ [89]	- -	- M □ [90]
VEGFR	- -	- -	- -	- M □ [64]	- M □ [64]	- M □ [64]	- -	H □ [11] -

Abbreviations: CSF-1, colony stimulating factor 1; EGF, epidermal growth factor; FGF-4, fibroblast growth factor 4; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-α, interferon alfa; IFN-1, interferon 1; IGF-1, insulin-like growth factor 1; IGF-2, insulin-like growth factor 2; IL-1, interleukin 1; IL-6, interleukin 6; LIF, leukemia inhibitory factor; PDGF-A, platelet-derived growth factor A; PDGF-B, platelet-derived growth factor B; SCF, stem cell growth factor; TGF-α, transforming growth factor alpha; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alfa; VEGF, vascular endothelial growth factor.

CSF-R, colony-stimulating factor 1 receptor; EGF-R, epidermal growth factor receptor; FGFR-1, fibroblast growth factor receptor 1; FGFR-2, fibroblast growth factor receptor 2; GM-CSF R, granulocyte-macrophage colony-stimulating factor receptor; IGF-I R, insulin-like growth factor I Receptor; IGF-II R, insulin-like growth factor II receptor; LIF-R, leukemia inhibitory factor receptor; PDGF-A R, platelet-derived growth factor A receptor; PDGF-B R, platelet-derived growth factor B receptor; SCFR, stem cell growth factor receptor; TGF-β R, transforming growth factor beta receptor; TNF R, tumor necrosis factor receptor; VEGFR, vascular endothelial growth factor receptor.

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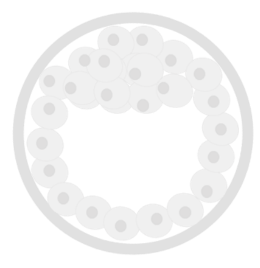


CHAPTER 2

AIMS OF THE THESIS

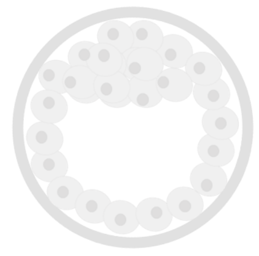
Ever since the start of bovine IVP in research labs, embryo culture has been performed in groups, whereas individual culture of bovine embryos has been a challenge. By contrast, in human settings embryos are mostly cultured individually since individual follow-up of the embryo is of utmost importance. However, it has been shown that preimplantation embryos are able to promote their own development by the production of autocrine factors. The presence of these autocrine factors in the medium when embryos are cultured in group lies at the basis of the embryos' superior development compared to single culture. The first papers describing autocrine communication between embryos *in vitro* date back to the early nineties, but almost 3 decades later only the tip of the iceberg of the involved signalling factors have been identified. However, a more in depth knowledge on the identity of autocrine factors would be helpful to further optimize media formulations for *in vitro* embryo culture of both bovine and human embryos. In bovine commercial IVP centres, embryos are cultured in small groups especially if one or a few oocytes can be obtained from a specific donor. The general aim of the present thesis was therefore to investigate and identify how inter-embryonic communication of bovine embryos occurs when cultured in group.

The specific scientific aims of the present thesis can be defined as follows:

1. To establish an accurate and reliable method to compare the developmental competence of bovine embryos cultured individually or in group
   - by developing a robust method in order to visualise 3 embryo quality parameters simultaneously: total cell number, inner cell mass ratio and apoptotic cell ratio (**Chapter 3**)
   - by developing an individual bovine embryo culture system, without the need of co-cultured somatic cells and similar to the human in vitro culture system, as a negative control system to compare with group culture (**Chapter 4**).

2. To revise the strengths and weaknesses of group culture by investigating possible negative effects of non-cleaved or delayed co-cultured embryos and defining which embryos profit the most from this group culture effect (**Chapter 5**).

3. To identify possible autocrine factors playing an important role in inter-embryonic communication during group culture. To this end, proteins secreted by bovine preimplantation embryos into the culture medium were analysed by means of tandem mass spectrometry (**Chapter 6**).



CHAPTER 3

EMBRYO QUALITY ASSESSMENT BY DIFFERENTIAL APOPTOTIC STAINING

Adapted from

Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining.

Wydooghe E, Vandaele L, Beek J, Favoreel H, Heindryckx B, De Sutter P and Van Soom A.

Analytical Biochemistry, 2011. 416 (2), 228-230.

1. Abstract

Several approaches have been described for differential staining of blastocysts, but these methods are often time consuming and unreliable. Here we describe a method for simultaneous differential staining and detection of apoptosis. The differential staining is based on the transcription factor CDX2, which is localized in the nucleus of trophectoderm (TE), cells but absent in the inner cell mass (ICM). Apoptosis is detected by staining of active caspase-3, a key player in several apoptotic pathways. This new approach represents a robust method to quantify simultaneously ICM/TE ratio and apoptotic cell ratio in bovine, murine, porcine and human blastocysts.

2. Introduction

Embryo morphology assessment is the most commonly used embryo quality parameter, albeit imperfect (Van Soom, et al. 2003). The first differentiation event is the segregation between the trophectoderm (TE) cells and the inner cell mass (ICM), which occurs at the morula stage and continues during the blastocyst stage. At about the same time, poor quality embryos may exhibit various features of apoptosis, such as activation of caspases, DNA fragmentation and nuclear fragmentation. Therefore, total cell number (TCN), inner cell mass/trophectoderm (ICM/TE) ratio and apoptotic cell ratio (ACR) offer important additional information for the evaluation of the quality of individual embryos (Fouladi-Nashta, et al. 2005). TCN and ICM/TE ratio can typically be evaluated in the same embryo by so-called differential staining methods for which various protocols have been described. Differential staining was first established in mice (Handyside and Hunter 1984), and later on in human and bovine blastocysts (Hardy, et al. 1989, Van Soom, et al. 1996). This method relied on the characteristic of ICM-cells having a compact network of tight junctions, which protect them from complement-mediated cell-lysis so that only TE-nuclei are stained with membrane impermeant polynucleotide-specific fluorochromes as Propidium iodide. Afterwards all nuclei are stained with a membrane permeant DNA stain as Hoechst 33342; like this the blue ICM-cells (Hoechst) can be distinguished from the pink TE-nuclei (Hoechst and Propidium iodide) (Handyside and Hunter 1984, Hardy, et al. 1989). Other differential staining methods were based on selective TE-cell permeabilisation by chemical techniques (Fouladi-Nashta, et al. 2005, Thouas, et al. 2001). The most important disadvantage of both techniques is the use of unfixed cells, which requires immediate staining of the embryos. Furthermore, both techniques proved very hard to repeat due to various technical difficulties. A way to circumvent selective permeabilisation of the TE-cells is to include an exclusive marker for TE-cells in the staining protocol. The transcription factor CDX2 is one of the earliest factors essential for the formation and

differentiation of TE-cells and its expression in the preimplantation mouse embryo is restricted to TE-cells (Beck, et al. 1995). This unique expression of CDX2 protein in the TE is evident in cattle (Kuijk, et al. 2008), pigs (Kuijk, et al. 2008), humans (Adjaye, et al. 2005) and primates (Sritanaudomchai, et al. 2009) and makes it a valuable marker to be included in a new differential staining protocol.

Besides ICM/TE ratio, apoptotic cell ratio (ACR) is a second important embryo quality marker. The presence of apoptotic cells is often visualised by fluorescent staining techniques. At present, terminal deoxynucleotidyl transferase-dUTP nick end labelling (TUNEL) assay to visualise DNA fragmentation is the most widely used technique for apoptosis detection in embryos (Byrne, et al. 1999; Gjörret, et al. 2003 & 2007; Vandaele, et al. 2006; Pero, et al. 2018). Likewise, double strand breaks in DNA are visualised by means of enzymatic labelling of the 3' end of oligonucleosome fragments. However, this technique is considered to lack specificity because necrotic nuclei may also show positive upon TUNEL staining (Graslkraupp, et al. 1995). Whereas DNA fragmentation visualises late stage apoptosis, the early stage of apoptosis can be visualised by labeled Annexin-V, identifying the flip of phosphatidylserine from the inner to the outer leaflet of the plasma membrane (Bakri, et al. 2016). Another approach, is to visualise direct on indirectly proteins, playing a specific role in the apoptotic pathways as e.g. caspases. Active caspase-3 is an executioner caspase, playing a central role in all apoptotic pathways: the receptor-ligand mediated (extrinsic), the mitochondrial (intrinsic) and the endoplasmic reticulum dependent pathway (Earnshaw, et al. 1999). Double immunofluorescent staining using an anti-caspase 3 antibody (Gjörret, et al. 2007) or direct immunofluorescence with fluorescent labeled caspase inhibitors have successfully been used (Spanos, et al. 2002). When combined with a DNA staining to visualize general apoptotic nuclear morphology, active caspase-3 detection in the cytoplasm is considered one of the best methods to evaluate apoptosis in embryos and tissues (Gjörret, et al. 2007).

Concurrent assessment of apoptosis and ICM/TE ratio in mammalian embryos would be a valuable technique to assess embryo quality. This study presents a useful and thorough protocol for a differential apoptotic staining based on CDX2 and active caspase-3 proteins.

3. Materials and methods

Bovine (Vandaele, et al. 2010), porcine (Bijttebier, et al. 2008), murine (Heindryckx, et al. 2002) and human (O'Leary, et al. 2011) embryos were obtained by routine *in vitro* production methods. Blastocysts were fixed in 4% paraformaldehyde for 20 minutes at room temperature (RT) and stored

in phosphate buffered saline (PBS) containing 0.5% Bovine Serum Albumin (BSA) (Sigma-Aldrich, Belgium) (PBS-BSA) at 4°C until the staining was performed.

The blastocysts were incubated overnight in 0.5% Triton X-100 (Sigma-Aldrich, Belgium) and 0.05% Tween-20 (Sigma-Aldrich, Belgium) in PBS at 4°C for permeabilisation. Except, for human and porcine blastocysts 1.5% Triton X-100 and 0.15% Tween-20 in PBS was used. Subsequently, the blastocysts were washed 3 times for 2 minutes in PBS-BSA. The second day, DNA of the blastomeres was denatured by exposure to 2N HCl for 20 minutes followed by 100 mM TrisHCl (pH= 8.5) for 10 minutes at RT. After denaturation and washing (3 times 2 minutes in PBS-BSA), the embryos were transferred to the blocking solution. Blocking occurred overnight in 10% Goat Serum (Invitrogen, Merelbeke, Belgium) and 0.05% Tween-20 in PBS at 4°C. Embryos serving as negative controls remained in blocking solution. The test embryos were washed and incubated in the ready to use primary CDX2 antibody (Biogenex, San Ramon, USA) for 1 to 3 days at 4°C. The test embryos were washed 2 times for 15 minutes at RT and incubated overnight at 4°C in rabbit active caspase-3 antibody (0.768µg/ml in blocking solution, Cell Signalling Technology, Leiden, The Netherlands). After another wash step (2 times 15 minutes), test embryos and negative controls were transferred to goat anti-mouse Texas Red antibody (20µg/ml in blocking solution, Molecular Probes, Merelbeke, Belgium) for either 1 hour at RT or 1-3 days at 4°C. The embryos were washed twice and incubated in goat anti-rabbit Fluorescein isothiocyanate (FITC) antibody (10µg/ml in blocking solution, Molecular Probes, Merelbeke, Belgium) for 1 hour at RT. After two wash steps of 15 minutes, the embryos were transferred to nuclear stain, Hoechst 33342 (50µg/ml in PBS/BSA, Molecular Probes, Merelbeke, Belgium) for 10 minutes at RT. After washing, embryos were mounted on a glass slide in a small drop of DABCO (maximal 2 embryos per slide) surrounded by 4 small vaseline drops and a cover slip was put on top. Likewise the 3D structure of the embryos remained intact despite the cover slip.

4. Results

Pictures of the embryos were taken the next day by fluorescence microscopy (Figure 1), with a U-M3DAFITR (Olympus) filter cub, a triple band pass filter for DAPI, FITC and Texas Red. The staining showed a perfect distinction between ICM and TE-cells and clear detection of apoptotic cells by caspase positivity in the cytoplasm.

In order to be able to count the TCN, number of TE-cells and the number of apoptotic cells, as is performed in chapter 4, 5 and 6 of this thesis, a fluorescent microscope (Leica DM 5500) was used with 3 different filters to visualise Hoechst, the Texas Red positive cells and the FITC positive cells

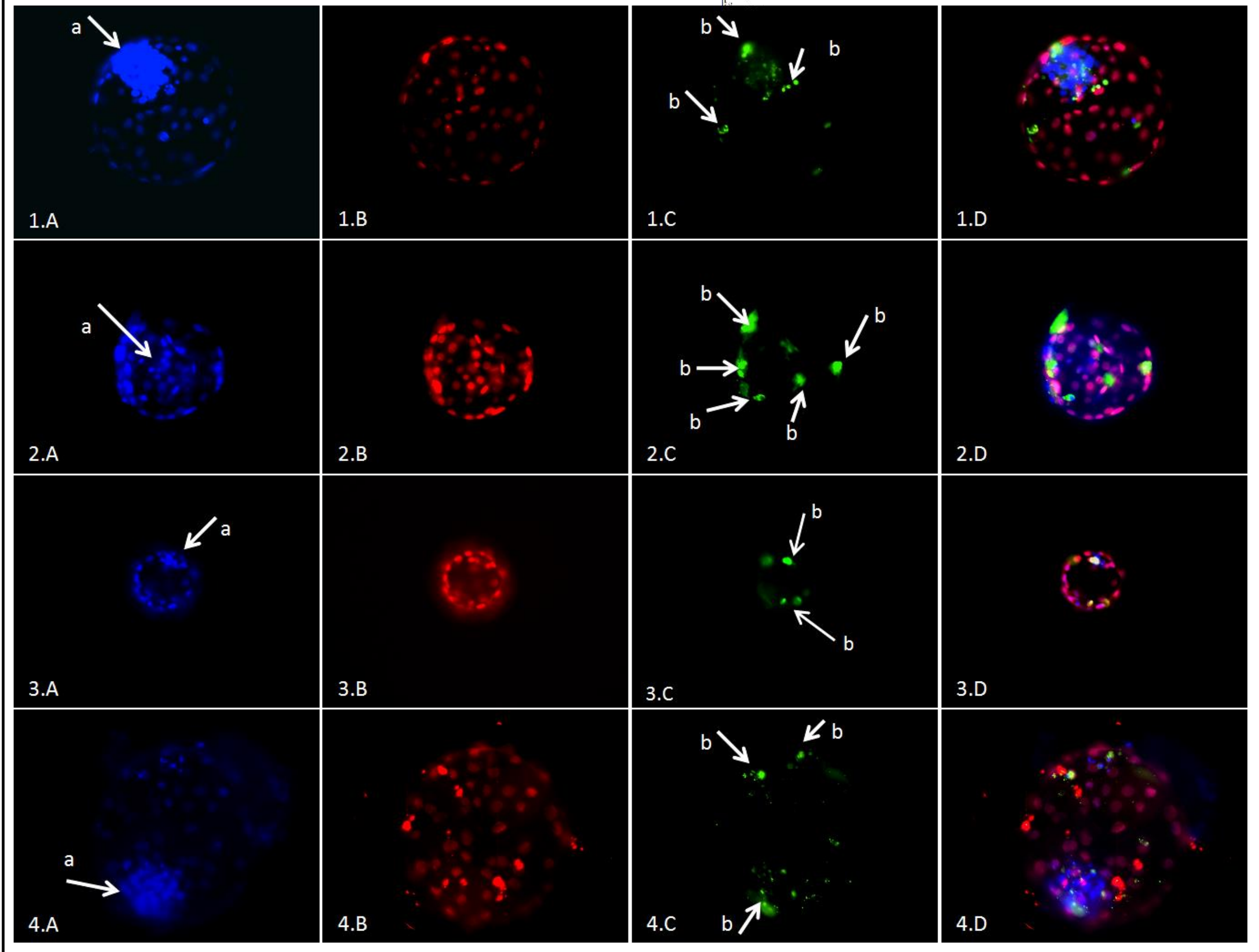


Figure 1. *Differential apoptotic staining of bovine (1.A-1.D), human (2.A- 2.D), murine (3.A-3.D) and porcine (4.A-4.D) blastocysts: both TE and ICM (arrow a) nuclei were stained with Hoechst (1.A –4.A). The CDX2 antibody was indirectly labelled with Texas Red resulting in a red fluorescent signal in TE cells only (1.B –4.B). The apoptotic cells (arrow b) show a green fluorescent signal because the anti-caspase 3 antibody was indirectly labelled with FITC (1.C – 4.C). An overlay is given in 1.D – 4.D. (20x magnification).*

separately. Since the 3D structure remained, the total cell number can be counted by scrolling through the embryo and counting all Hoechst positive cells. Hoechst positive cells were counted twice, and the difference between the two counts should not exceed 10 cells. Afterwards, the TE cells were counted similarly using the Texas Red filter. And finally, apoptotic cell number was counted as the number of cells with a FITC positive cytoplasm (active caspase-3) with concurrent general apoptotic nuclear morphology (as described by Gjörret et al., 2007).

5. Discussion

We established and validated a new, easy and repeatable method for differential staining of embryos of several mammalian species, with concurrent detection of apoptosis. The most important advantage compared to earlier described methods, is that zona intact embryos can be fixed in paraformaldehyde and stored in PBS-BSA until the moment of staining. This protocol also averts the technical difficulties of complement mediated cell-lysis on unfixed zona-free blastocysts or chemically based partial permeabilisation of the TE-cells of unfixed zona-intact blastocysts. Thouas (2001) described that the time of chemical permeabilisation should be very short (a few seconds) to be efficient, with too long exposure leading to abnormal staining patterns and that the optimal timing of permeabilisation has to be redefined for each species and for each laboratory setting (Thouas, et al. 2001). The CDX2 protein is as transcription factor very closely connected to the DNA of the blastomeres. The nucleus contains a HCl-soluble fraction, which consists of histone and non-histone material. We hypothesize that denaturation of this fraction using HCl is necessary to expose the CDX2 epitopes (Robbins and Borun 1967). Furthermore, extreme pH-values (around 2 and 10) appear to enhance nuclear antigen retrieval for monoclonal antibodies, with a basic solution being more efficient (Abdalla, et al. 1993, Kim, et al. 2004). For this double immunofluorescent staining, the most commonly used basic buffer to enhance antigen retrieval, TrisHCl, worked perfectly (D'Amico, et al. 2009).

Human and porcine blastocysts need a higher concentration of Triton X-100 for permeabilisation. With this refinement, the protocol worked flawlessly for human blastocysts, but it was still difficult to obtain repeatable staining in porcine blastocysts. This may be due to the fact that pig embryos have a thick zona pellucida which is about 16 µm in diameter, as opposed to the murine zona pellucida of 5 µm (Van Soom, et al. 2010) or to the poor quality of some *in vitro* produced pig blastocysts (Papaioannou and Ebert 1988).

Concurrent assessment of ICM/TE ratio and ACR in one single embryo is of paramount importance to minimize sample size and costs of experiments in all mammalian species. The current

staining can also successfully be applied in human embryos, which are very scarce, providing researchers a tool to gain as much information as possible on the quality of a single embryo. In conclusion, this double immunofluorescent staining represents an easy, repeatable and robust method for differential apoptotic staining of blastocysts which can be applied in bovine, murine, human and porcine blastocysts of good quality.

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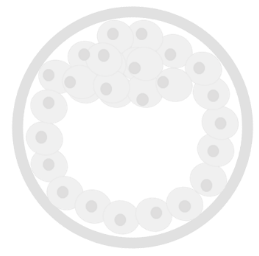

CHAPTER 4

STEPWISE FROM GROUP CULTURE TO INDIVIDUAL CULTURE

Adapted from

Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture.

Wydooghe E, Heras S, Dewulf J, Piepers S, Van den Abbeel E, De Sutter P, Vandaele L, Van Soom A. Reproduction, Fertility and Development. 2014. 26(5): 717-24.

1. Abstract

Individual culture of bovine embryos is usually associated with low blastocyst development. However, during preliminary experiments in our laboratory we observed high blastocyst development after individual embryo culture in a serum-free culture system. We therefore hypothesized that serum has a negative effect on embryos cultured individually whereas embryos in groups can counteract this. First, we determined whether the timing of removal of serum (during maturation or culture) had an influence on individual embryo development. The results clearly showed that removal of serum during embryo culture was the main contributing factor since high blastocyst development was observed after individual culture in synthetic oviductal fluid supplemented with bovine serum albumin (BSA) and insulin, transferrin and selenium (ITS), independent of the maturation medium. Second, we investigated whether an individual factor of the ITS supplement was essential for individual embryo development. We demonstrated that repeatable high blastocyst percentages were due to the synergistic effect of ITS. Finally, we investigated if a group-culture effect can still be observed under serum-free conditions. Group culture generated blastocysts with higher total cell numbers and less apoptosis. These data show that individual culture in serum-free conditions leads to high blastocyst development, but group culture still improves blastocyst quality.

2. Introduction

In its natural environment, a developing mammalian embryo is subjected to a complex communication with the female genital tract by autocrine, paracrine and endocrine factors (O'Neill 2008). Likewise, the oviduct and the uterine epithelial cells are able to provide the dynamic and elastic surroundings on which the embryo depends for its development and survival (Buhi, et al. 2000, Scotchie, et al. 2009). Since many studies on the *in vitro* development of preimplantation embryos pointed towards the importance of autocrine communication between embryos *in vitro* as replacement for the complex communication *in vivo*, group culture has been adopted by many research groups as the routine procedure for animal embryo culture (O'Neill 1997, 2008, O'Neill, et al. 2012, Paria and Dey 1990). However, for scientists, the development of an individual culture system would be an important tool to gain more insight into the requirements and metabolism of preimplantation bovine embryos cultured *in vitro*, but also in commercial settings it would beneficial, since donor cows often have low numbers of oocytes. Still, most attempts to introduce individual culture in cattle embryos have shown a reduction in development from a typical 25–35% blastocysts in group culture (Lonergan 2007) to less than 10% in individual culture (Donnay, et al. 1997, Goovaerts, et al. 2009, O'Doherty, et al. 1997).

Foetal bovine serum (FBS) is typically added during *in vitro* production of bovine embryos to supply growth factors and energy sources. Although several authors have demonstrated the beneficial effect of FBS supplementation, downsides are that the composition of FBS is largely unknown and varies between batches, there is a contamination risk and it may increase the incidence of large offspring syndrome (Lazzari, et al. 2002). Lonergan et al. (1996) showed that FBS in maturation medium can be replaced by epidermal growth factor (EGF) without changes in blastocyst development, hatching rate and total cell number. During the last decade serum-free semi-defined culture media have been introduced into bovine embryo culture (George, et al. 2008, Krisher, et al. 1999, Rizos, et al. 2003). Interestingly, while attempting to culture bovine embryos under serum-free conditions, serendipitous observations were made in our laboratory that the very simple act of using serum-free culture medium based on bovine serum albumin (BSA) and insulin, transferrin and selenium (ITS), unexpectedly led to high blastocyst development in individual culture of bovine embryos (Vandaele, et al. 2010). In the past, this combination of BSA and ITS has been described as a valuable alternative for serum supplementation, not only for somatic cell culture (Barnes and Sato 1980a, b) but also for *in vitro* production of bovine embryos (Bowles and Lishman 1998, George, et al. 2008, Palasz and Thundathil 1998, Shamsuddin, et al. 1993). George et al. (2008) showed that synthetic oviductal fluid (SOF) with BSA and ITS can also be used to culture bovine embryos in small groups of five embryos.

Therefore, we hypothesized that serum in the culture medium has a negative effect on the development of individually cultured bovine embryos and this effect disappears when serum is removed from the medium. For that reason, it was important to find out whether the removal of serum from the medium was equally important during oocyte maturation or during embryo culture. Second, we also aimed to identify which factor of the ITS combination was the most important in supporting embryo development during individual culture. In a last part, we investigated whether group culture in the BSA–ITS-based serum-free culture system adds an additional beneficial effect to the quality of the generated blastocysts.

3. Materials and methods

3.1 Media and reagents

Basal medium eagle amino acids, minimal essential medium non-essential amino acids (100×), Tissue culture medium (TCM-199), kanamycin and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium). Foetal bovine serum was obtained from Greiner Bio-one (Wemmel, Belgium), and all other components were obtained from Sigma (Schnelldorf, Germany). All media were filter-sterilized (0.22 µm; Pall Corporation, Ann Arbor, MI, USA) before use.

3.2 *In vitro* embryo production

Bovine embryos were produced by previously-described routine *in vitro* methods (Vandaele, et al. 2006). Briefly, bovine ovaries were collected at the local slaughterhouse and processed within 2 h. The ovaries were washed three times in warm physiological saline supplemented with kanamycin (25 mg/ml). The oocytes were aspirated from follicles between 4 and 8 mm diameter and matured in groups of 60 in 500 µL modified bicarbonate-buffered TCM-199 supplemented depending on the experimental set-up either with 20% heat-inactivated FBS, 50 µg/ml gentamycin, 0.4 mM L-glutamine, 0.1 mM cysteamine and 2 mM Na-pyruvate (serum conditions) or 50 µg/ml gentamycin and 20 ng/ml EGF (serum-free conditions) for 22 h at 38.5°C in 5% CO_2 in air. Frozen–thawed bovine spermatozoa were separated over a Percoll gradient (45 and 90%; GE Healthcare Biosciences, Uppsala, Sweden) and washed afterwards. The final sperm concentration of 1×10^6 spermatozoa/mL was adjusted in IVF–Tyrode's albumin–pyruvate–lactate (TALP), consisting of bicarbonate-buffered Tyrode solution, supplemented with BSA (Sigma A8806; 6 mg/ml) and heparin (25 µg/ml). The matured oocytes were washed in 500 µL IVF–TALP and incubated with spermatozoa for 21 h. Because of the dark cytoplasm of bovine oocytes, fertilisation rate cannot be evaluated using light microscopy, but requires Hoechst staining to visualise the pronuclei. As described by Thys *et al.* (2009), the IVF procedure described here resulted in a fertilisation rate (two pronuclei seen) of 77.1% and a penetration rate (at least two pronuclei seen) of 83.6%, evaluated after Hoechst staining in similar experiments (3 replicates). After removal of the excess spermatozoa and cumulus cells (vortex), only the zygotes without adhering cumulus cells were transferred to synthetic oviductal fluid supplemented with essential and non-essential amino acids (SOFaa) and, depending on the experimental set-up, supplemented with either 5% FBS or 0.4% BSA (Sigma) and ITS (5 µg mL–1 insulin + 5 µg mL–1 transferrin + 5 ng mL–1 selenium). Culture occurred either in groups of 25 in 50-µL drops or individually in drops of 20 µL, both covered with mineral oil and incubated at 38°C in 5% CO_2, 5% O_2 and 90% N_2. To avoid bias, both group culture and individual culture occurred in Petri dishes (BD Biosciences, Erembodegem, Belgium). For group culture, 4 drops of 50 µl were placed in one Petri dish and covered by 7.5 ml of mineral oil, whereas for individual culture per Petri dish 17 drops of 20 µl were covered by 7.5 ml of mineral oil (apart together strategy). Evaluation of embryo development occurred at 45 hours post insemination (hpi) as the percentage of cleaved embryos out of presumed zygotes and at 168 and 192 hpi as the percentage of blastocysts out of presumed zygotes. At 192 hpi, hatching rate was evaluated as the percentage of hatching or hatched blastocysts compared with the total number of blastocysts.

3.3 Experiment 1: When is the removal of serum the most important: during maturation or culture?

The first experiment was designed to determine whether the removal of serum either in the maturation medium, the culture medium or both is important for the development of individually cultured embryos in cattle. As previously described, oocytes (n = 1320; three replicates) were matured either in serum or in serum-free TCM-199. After fertilisation, half of the zygotes of each group were cultured individually in serum-containing and the other half in serum-free SOFaa in order to obtain four experimental groups: (1) FBS/FBS, (2) FBS/BSA+ITS, (3) EGF/FBS and (4) EGF/BSA+ITS. These four experimental groups have been compared simultaneously with the control, cultured in groups in serum conditions (GROUP).

3.4 Experiment 2: Does ITS contain the essential factor for individual embryo culture?

This experiment was designed to find out which one, if any, of the components of ITS is crucial for individual bovine embryo development. Oocytes (n = 749, three replicates) were matured serum-free and cultured individually in four different groups: in SOFaa supplemented with either (1) BSA + 5 µg/ml insulin, (2) BSA + 5µg/ml transferrin, (3) BSA + 5 ng/ml selenium or (4) BSA alone. Simultaneous individual culture in SOFaa supplemented with BSA and ITS was performed as a control.

3.5 Experiment 3: Does the 'group-culture effect' remain in serum-free conditions?

The last experiment was designed to determine whether a beneficial effect of being cultured in groups remains in serum-free conditions. Oocytes (n = 1130, three replicates) were at random assigned to maturation in serum or in serum-free conditions. After fertilisation, half of the zygotes matured in serum conditions were subsequently cultured individually and the other half in groups of 25 in serum-containing culture medium. The zygotes matured in serum-free conditions were also allocated to individual or group culture, both cultured in serum-free medium. In this way, four experimental groups were obtained: (1) GR FBS/FBS, (2) IND FBS/FBS, (3) GR EGF/BSA+ITS and (4) IND EGF/BSA+ITS.

3.6 Differential apoptotic staining

At 192 hpi all identified blastocysts were fixed in 2% paraformaldehyde during 20 min and subsequently subjected to a differential apoptotic staining in order to assess embryo quality (Wydooghe, et al. 2011). This is a double-immunofluorescent staining against CDX2, a transcription

factor uniquely expressed in the trophectoderm cells, and against active caspase-3, which plays a central role in all apoptotic pathways. This staining is further combined with a Hoechst nuclear stain. This allowed us to evaluate simultaneously three important embryo quality parameters: the total cell number (TCN), proportion of inner cell mass (ICM) relative to the TCN (ICM ratio) and apoptotic cell ratio (ACR), which is the percentage of apoptotic cells relative to the TCN.

3.7 Statistical analysis

All experiments were repeated three times. To analyse the developmental data binary-logistic mixed-regression models using first-order penalised quasi-likelihood algorithms were fitted in MLwiN 2.02 (Centre of Multilevel Modelling, Bristol, UK) with replicate as random effect and group as fixed effect. Significance was assessed at $P < 0.05$. Data concerning blastocyst quality (i.e. TCN, ACR and ICM ratio) were analysed using a mixed-model analysis of variance, with group as fixed factor and replicate as random factor. When the data were not normally distributed, a log transformation was applied. Data concerning blastocyst quality are expressed as means ± standard error of the mean (s.e.m.). Differences at $P < 0.05$ were considered to be significant.

4. Results

4.1 Experiment 1: When is the removal of serum the most important: during maturation or culture?

At 45 hpi embryos matured and cultured in serum-free conditions (EGF/BSA+ITS) showed higher embryonic cleavage compared with the control group; the other experimental groups showed no differences compared with the control (GROUP, 69.2 ± 2.94; FBS/FBS, 72.9 ± 2.77; FBS/BSA+ITS, 73.2 ± 2.70; EGF/FBS, 71.6 ± 2.78; EGF/BSA+ITS, 83.0 ± 2.24). At 168 hpi blastocyst development was similar for the embryos cultured individually in serum-free culture medium (FBS/BSA+ITS and EGF/BSA+ITS) compared with the control group independent of maturation condition (Table 1). The embryos cultured individually in serum-containing medium (FBS/FBS and EGF/FBS) showed a lower blastocyst development than the control group ($P < 0.05$). Comparable differences were found on 192 hpi. Since no embryos reached the hatching stage when they were matured and subsequently cultured individually in serum conditions (FBS/FBS), we no longer accounted for this treatment for the statistical analyses of the hatching rate. We observed that fewer blastocysts reached the hatching stage in the treatments EGF/FBS and EGF/BSA+ITS compared with the control group. When embryos were matured

in serum conditions and cultured in serum-free medium (FBS/BSA+ITS) we observed a tendency towards a lower hatching rate (P = 0.09).

The TCN of the blastocysts collected at 192 hpi was lower for the treatment EGF/FBS compared with the control, but no differences were found for the other groups (Fig. 1). The ICM ratio was higher for all treatments compared with the control except for the treatment FBS/FBS, where no differences in ICM ratio were observed. The treatments EGF/ITS and FBS/ITS showed a higher absolute number of ICM cells, whereas the treatment EGF/FBS had fewer trophectoderm cells. No differences in apoptotic cell ratio (ACR) were observed compared with group culture in serum conditions for all embryos tested.

Table 1. Blastocyst development and hatching rate after individual culture (with serum present or absent during maturation and culture) as compared with group culture in serum conditions.

Culture system	IVM	IVC	No. of presumed zygotes	Blastocyst yield (%)		
				168 hpi[A]	192 hpi[A]	Hatching[B]
Group	FBS	FBS	247	21.9 ± 2.63	23.1 ± 2.68	31.6 ± 6.15
Individual	FBS	FBS	258	**8.1 ± 1.69***	**9.1 ± 1.77***	0
		BSA + ITS	264	19.3 ± 2.41	25.3 ± 2.65	**19.1 ± 4.77****
	EGF	FBS	282	**7.6 ± 1.63***	**6.4 ± 1.51***	**5.9 ± 5.9***
		BSA + ITS	269	22.0 ± 2.47	30.9 ± 2.75	**14.9 ± 3.82***

[A] Blastocyst yield is expressed as the percentage of blastocysts at 168 hpi and 192 hpi respectively out of the number of presumed zygotes.
[B] Hatching rates are expressed as the percentage of hatching or hatched blastocysts at 192 hpi compared with the total number of blastocysts.
Data are expressed as mean ± s.e.m. Differences at P < 0.05 were considered to be significant and are marked with an asterisk. Differences at P < 0.1 were considered as a trend and are marked with a double asterisk.

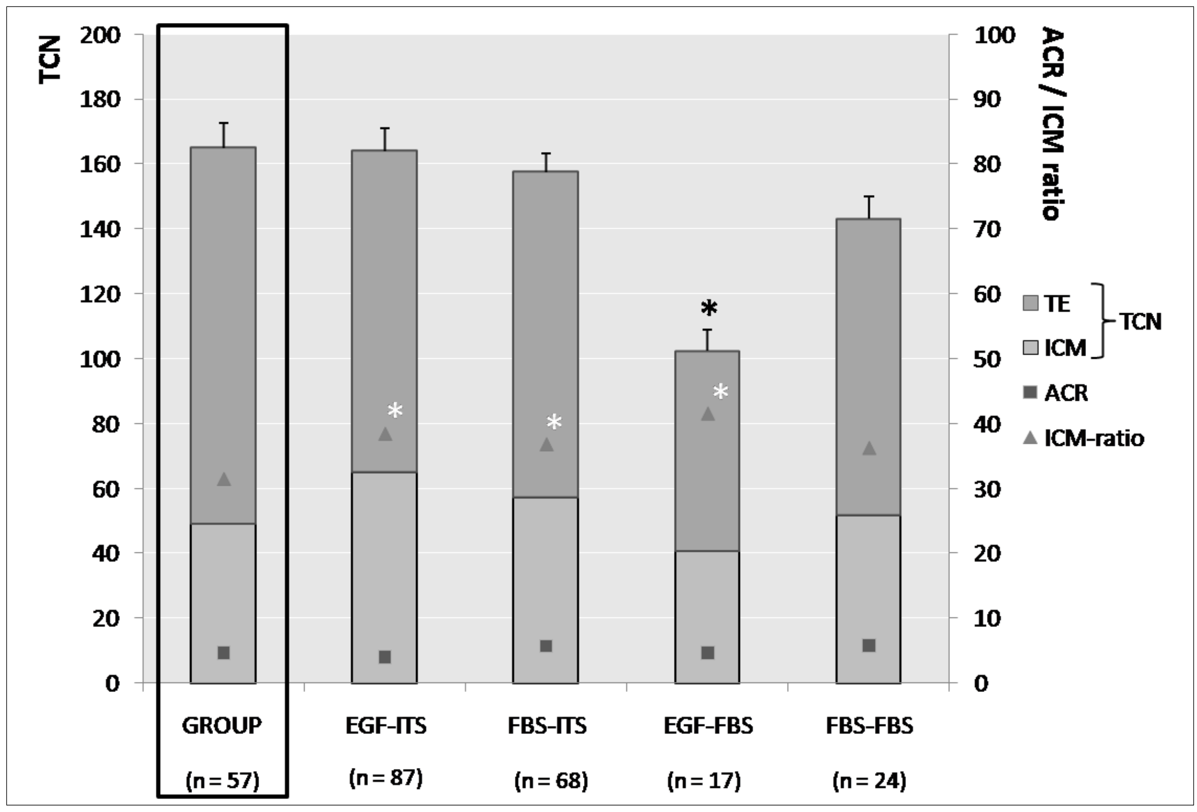

Figure 1. *Total cell number (TCN) as the sum of trophectoderm cells (TE) and cells allocated to the inner cell mass (ICM), ICM ratio and apoptotic cell ratio (ACR) of blastocysts collected at 192 hpi after differential apoptotic staining. Embryos were cultured individually in the presence or absence of serum in maturation and culture medium. Significant differences as compared with the control (P < 0.05) are marked with an asterisk.*

4.2 Experiment 2: Does ITS contain the essential factor for individual embryo culture?

The embryonic cleavage at 45 hpi was not different for all four experimental treatments compared with the control (data not shown). The supplementation of insulin or transferrin alone did not change the blastocyst development at 168 hpi compared with the control, but the absence of both of these components (supplementation with S or BSA only) lowered blastocyst development significantly (Table 2). At 192 hpi, however, no differences were observed anymore for all treatments compared with the control.

Concerning blastocyst quality, TCN was only lower in BSA treatment compared with the control with ITS (Figure 2). The ICM ratio was lower in all conditions compared with ITS, since the synergic effect of the three components of ITS was leading to blastocysts with the highest absolute number of ICM cells. Interestingly, ACR was higher for blastocysts produced in the presence of BSA only compared with the control.

Table 2. Influence of addition of single components of ITS on blastocyst development and hatching after individual culture in SOFaa with 0.4% BSA.

Component(s) added to embryo culture	No. of presumed zygotes	Blastocyst yield (%)		
		168 hpi[A]	192 hpi[A]	Hatching[B]
Insulin, transferrin and selenium	151	21.9 ± 3.36	29.1 ± 3.70	20.0 ± 5.96
Insulin	152	14.5 ± 2.85	25.5 ± 3.53	12.8 ± 5.35
Transferrin	146	13.0 ± 2.78	21.9 ± 3.42	13.8 ± 6.40
Selenium	153	9.8 ± 2.40*	18.3 ± 3.13	22.2 ± 8.00
None	150	12.2 ± 2.69*	20.9 ± 3.34	12.9 ± 6.02

[A] *Blastocyst yield is expressed as the percentage of blastocysts at 168 hpi and 192 hpi respectively out of the number of presumed zygotes.*

[B] *Hatching rates are expressed as the percentage of hatching or hatched blastocysts at 192 hpi compared with the total number of blastocysts.*

Data of separate components were compared with the combined addition of insulin, transferrin and selenium and are expressed as mean ± s.e.m. Differences at P < 0.05 were considered to be significant and are marked with an asterisk.

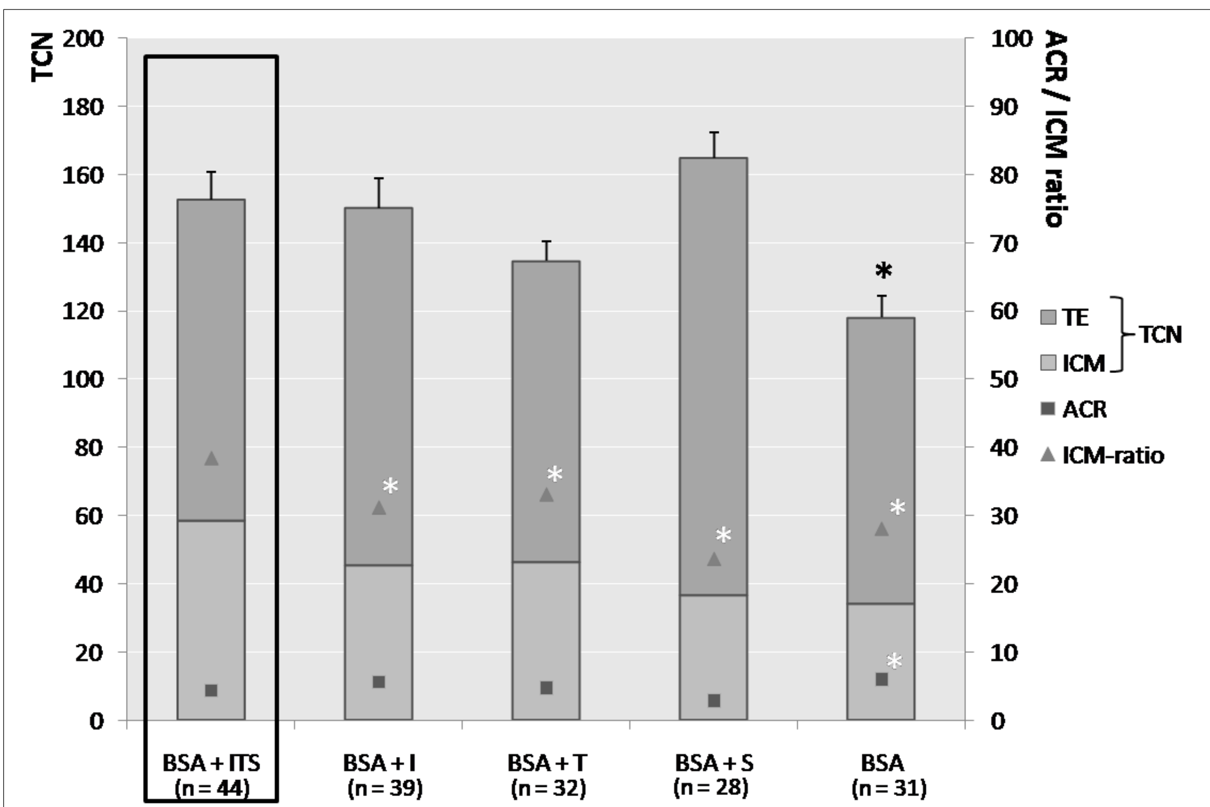

Figure 2. *Total cell number (TCN) as the sum of trophectoderm cells (TE) and cells allocated to the inner cell mass (ICM), ICM ratio and apoptotic cell ratio (ACR) of blastocysts collected at 192 hpi after differential apoptotic staining. Embryos were cultured individually in SOFaa with BSA, supplemented either with insulin, transferrin, selenium or none of these factors. Significant differences as compared with the control (SOFaa with BSA and ITS; P < 0.05) are marked with an asterisk.*

4.3 Experiment 3: Does the 'group-culture effect' remain in serum-free conditions?

Embryos cultured in groups or individually in serum-free conditions showed a higher embryonic cleavage compared with group culture in serum conditions (GR FBS, 63.7 ± 2.78; IND FBS, 71.2 ± 2.59; GR BSA+ITS, 74.5 ± 2.63; IND BSA+ITS, 83.2 ± 2.36). At 168 hpi blastocyst development in serum conditions was inferior in individual culture compared with group culture, whereas in serum-free conditions, group or individual culture yielded similar blastocyst development as the control group culture in serum (Table 3). Additionally, group and individual culture in serum-free conditions were compared; however, no differences were observed between those groups (after Bonferroni correction). The same differences were observed at 192 hpi. The hatching rate was lower in individual culture compared with group culture in serum conditions. Remarkably, the hatching rate was significantly lower for both group and individual culture in serum-free conditions compared with group culture in serum conditions, but no differences were seen between group and individual culture in serum-free conditions (after Bonferroni correction).

Regarding the quality of the obtained blastocysts (Figure 3), embryos cultured in groups showed a higher TCN compared with individually cultured embryos both in serum and serum-free conditions. Group-cultured embryos also had a lower ACR compared with their counterparts cultured individually. The ICM ratio was not different between group and individual culture.

Table 3. Blastocyst development and hatching rates of embryos cultured in groups or individually in serum and serum-free conditions.

Culture system	IVM	IVC	No. of presumed zygotes	Blastocyst yield (%)		
				168 hpi[A]	192 hpi[A]	Hatching[B]
Group	FBS	FBS	300	29.7 ± 2.64	31.2 ± 2.67	34.0 ± 4.89
Individual			306	7.8 ± 1.53*	12.4 ± 1.88*	2.6 ± 2.58*
Group	EGF	BSA + ITS	274	29.6 ± 2.76[a]	36.5 ± 2.91[a]	15.0 ± 3.57*[a]
Individual			250	24.0 ± 2.70[a]	35.2 ± 3.02[a]	12.5 ± 3.53*[a]

[A] *Blastocyst yield is expressed as the percentage of blastocysts at 168 hpi and 192 hpi respectively out of the number of presumed zygotes.*
[B] *Hatching rates are expressed as the percentage of hatching or hatched blastocysts at 192 hpi compared with the total number of blastocysts.*
Data were compared with the control, group culture in serum conditions, and differences which were significant (P < 0.05) are marked with an asterisk. Additionally, group and individual culture in serum-free conditions were compared; differences at P < 0.05 (after Bonferroni correction) are marked with different superscripts. Data are expressed as mean ± s.e.m

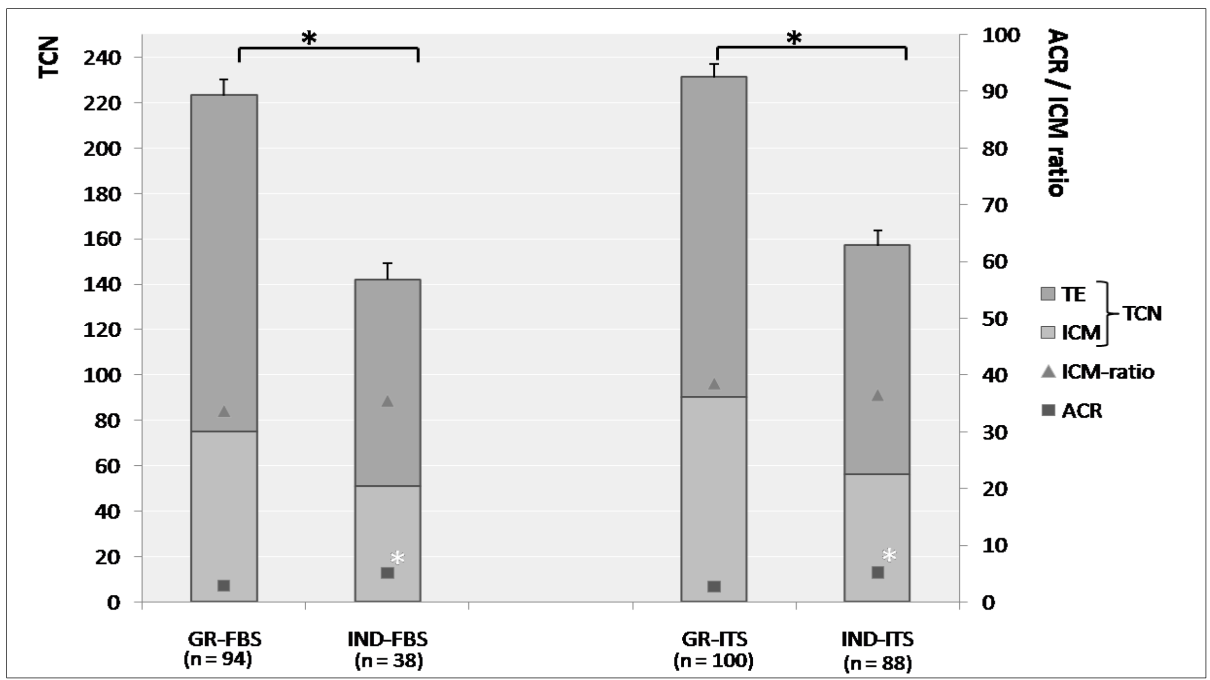

Figure 3. *Total cell number (TCN) as the sum of trophectoderm cells (TE) and cells allocated to the inner cell mass (ICM), ICM ratio and apoptotic cell ratio (ACR) of blastocysts collected at 192 hpi after differential apoptotic staining. Results pertain to embryos cultured in groups (GR) or individually (IND) in either serum (FBS) or serum-free conditions (ITS). Differences at P < 0.05 were considered to be significant and are marked with an asterisk.*

5. Discussion

In the present study, we demonstrated for the first time that in serum-free conditions equally high blastocyst development can be obtained both with individual culture and group culture; with the main advantage of group culture over individual culture leading to superior blastocyst quality in terms of higher total cell number and lower apoptotic cell ratio. We state that the inferior development of individually cultured bovine embryos compared with group culture, as described in former papers, is predominantly caused by the supplementation of serum to the culture medium. From our results, we can conclude that this inferior development can mainly be explained by the fact that in serum conditions embryos need to be cultured in groups to produce sufficient autocrine factors to revoke a negative factor present in serum, which has also been noticed in the past (Donnay, et al. 1997, Goovaerts, et al. 2009, O'Doherty, et al. 1997). Another important aspect in this experimental set-up is the embryo density, expressed as the relative amount of medium per embryo. In our set-up, individual embryos were exposed to a relatively higher amount of medium (20 µL per embryo) compared with embryos cultured in groups (2 µL per embryo), with a logical consequence that the individual embryo is not a match for the adverse effect of serum in culture medium. To the best of our knowledge, there are no papers describing acceptable bovine embryo development after individual

culture in serum-containing medium. The only exception to date is a study of Carolan et al. (1996); they could obtain high blastocyst development after individual culture in serum-containing medium. No other studies describe comparable results after that, so probably the favourable outcome was related to the beneficial qualities of that particular batch of serum. Recently, Goovaerts et al. (2009) showed that cattle embryos can be cultured individually in serum conditions when a co-culture with autologous cumulus cells is used. This proves also that the concentration of autocrine factors produced by individual embryos is not high enough; they need the help of other secreting cells to overcome the adverse effect of serum. However, even when embryos are cultured in groups, an inhibiting effect on embryo development because of serum supplementation is described, since fewer embryos complete the first cleavage division (Pinyopummintr and Bavister 1991). This finding can be supported by the results of Experiments 1 and 3, where we observed a lower embryonic cleavage in serum conditions compared with serum-free conditions. Furthermore, our results confirm that serum supplementation in group culture clearly influences development in a biphasic manner as described by Pinyopummintr and Bavister (1991), since no differences could be found in blastocyst development on 168 and 192 hpi compared with serum-free conditions. Remarkably, serum supplementation significantly facilitated hatching of the blastocysts formed at 192 hpi. It has been shown before that bovine embryos produce plasminogen activator, a protease that can activate plasminogen, a zymogen present in serum, to the enzyme plasmin. By this conversion plasmin can cause a weakening of the zona pellucida to simplify hatching (Kaaekuahiwi and Menino 1990, Menino and Williams 1987).

When embryos are cultured in serum-free conditions, based on BSA and ITS, we demonstrated that for individually-cultured embryos blastocyst development was comparable to that obtained after group culture. This is in agreement with Hagemann et al. (1998) and Goovaerts et al. (2012), who could also obtain acceptable blastocyst development when cattle embryos were cultured individually in a semi-defined medium; however, no direct comparison with group culture was done in those studies. On the other hand, an important aspect in the experimental set-up of those studies is that a relatively high percentage of BSA was supplemented to the culture medium: Hagemann et al. (1998) used SOF with 3% BSA, whilst Goovaerts et al. (2012) used SOF with 2% BSA and ITS. From a dose-response curve with BSA (data not shown) we observed that the best embryo development and hatching rates were obtained with low amounts of BSA (0.4% or 0.8%). The fact that a low concentration of albumin is more favourable for embryo development has also been described in the mouse (Otsuki, et al. 2013).

For us, it was important to know whether one of the components of ITS plays a dominant role in the support of individual embryos. Insulin is a peptide hormone that plays an important role in the uptake of glucose in adult life and has growth promoting effects on preimplantation embryos of several

species (Kane, et al. 1997). It has been described that the effect of insulin on rabbit embryos is not working through glucose metabolism but instead insulin is acting as a growth factor binding to its receptor, providing growth-promoting and anti-apoptotic functions to the embryo (Navarrete Santos, et al. 2000) and the same effects have been described for bovine embryos (Augustin, et al. 2003). Furthermore, insulin has also been described to increase the ICM ratio in mouse embryos (Harvey and Kaye 1990); however, we could not confirm this for bovine embryos. Transferrin and selenium are both essential trace elements. Transferrin is an iron-binding serum protein that can sequester free traces of iron to prevent highly toxic radical formation and is described to help overcome the *in vitro* two-cell block of mouse embryos (Nasresfahani and Johnson 1992). Transitional metals such as iron can cause cell damage by catalysing the formation of reactive oxygen species. Bowles and Lishman (1998) described earlier that the addition of transferrin alone or in combination with insulin increased bovine blastocyst development of embryos cultured in groups. Selenium is also able to protect cells from oxidative damage because of its activity in the glutathione–peroxidase pathway (Barnes and Sato 1980a). An important conclusion from our data is that the addition of insulin or transferrin to the culture medium is crucial to support individual embryo development until 168 hpi. Without insulin or transferrin addition to the culture medium, we observed a delayed cell allocation to ICM resulting in lower blastocyst development at 168 hpi; however, the differences disappeared at 192 hpi. In order to obtain high blastocyst development and top-quality embryos with the highest ICM ratio, the synergism of the three components of ITS is needed. Remarkably, we could still observe a group-culture effect in serum-free culture medium based on BSA and ITS, typically generating blastocysts with higher total cell numbers and less apoptosis. This indicates that group culture can still play a role in serum-free conditions, probably due to the release of autocrine factors by the embryos. The identity of these autocrine embryonic factors is for the greater part unknown, although one might speculate that likely candidate molecules are basic fibroblast growth factor, insulin-like growth factor 1 and 2, interferon, platelet-derived growth factor alfa and transforming growth factor beta, since their mRNA has been identified in bovine preimplantation embryos (Watson, et al. 1992); however, the secretion of these factors by bovine embryos still needs to be confirmed. Recently-developed techniques such as liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) offer an important tool to unravel the identity of the autocrine factors that play a significant role in the embryo–embryo dialogue at the protein level. At present, we are optimising the technique for further qualification and quantification of the proteins synthesised and secreted by the individual embryo into the culture medium, the so-called secretome. This will hopefully reveal important information about the physiology of the mammalian embryo and could lead to the development of potential biomarkers for embryo viability. The identified proteins could be used as non-invasive parameters in addition to embryo morphology or to further optimise the serum-free individual culture system.

In conclusion, we showed for the first time that using a serum-free culture medium, based on BSA supplemented with insulin, transferrin and selenium leads to high and repeatable blastocyst development after individual culture of bovine embryos. The development of a semi-defined individual culture system is not only an important tool for research laboratories and practitioners working with bovine IVF, in addition this study upgraded the value of the bovine embryos as a model for human IVF (Hansen 2010, Van Soom, et al. 2011), since in most European human IVF centres individual embryo culture is the standard procedure. However, further research will be needed to boost the embryo quality of bovine embryos in this serum-free individual system to the same level or above that of embryos cultured in groups.

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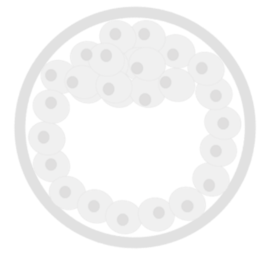

CHAPTER 5

STRENGTHS AND WEAKNESSES OF GROUP CULTURE

Adapted from

Individual commitment to a group effect: strengths and weaknesses of bovine embryo group culture.

Wydooghe E, Vandaele L, Piepers S, Dewulf J, Van den Abbeel E, De Sutter P and Van Soom A. Reproduction. 2014. 148: 519-529.

1. Abstract

Recently, new culture devices such as Corral® and Primo Vision® dishes have been designed for culture of human embryos to allow the combination of group culture plus follow-up of individual embryos. Bovine inseminated oocytes were allocated to Primo Vision® dishes, Corral® dishes, individual culture, or classical group culture. Blastocyst development in Primo Vision® dishes was similar to classical group culture (34.3% and 39.0% respectively), and better than Corral® dishes or individual culture (28.9% and 28.5% respectively). In Primo Vision® dishes, a higher number of 'slow' embryos developed to the blastocyst stage compared to their individually cultured counterparts, while no differences were observed for 'fast' embryos. 'Slow' embryos in a 'standard drop' had a higher chance of becoming a blastocyst compared to individual culture (OR: 2.3), whereas blastulation of 'fast' embryos was less efficient in a 'delayed drop' than in individual culture (OR: 0.3). The number of non-cleaved embryos in Primo Vision® dishes did not negatively influence blastocyst development. Likewise, removing non-cleaved embryos (NC removed) and regrouping the cleaved embryos afterwards (ReGR), did not affect blastocyst development and quality compared to group culture in Primo Vision® dishes (CTRL: 31.6%, NC removed:29.3%, ReGR: 29.6%). The experiments showed that group culture of bovine embryos in Primo Vision® dishes is superior to individual culture, primarily because of the higher blastocyst rate achieved by slow embryos. Non-cleaved or arrested embryos do not hamper the ability of co-cultured bovine embryos to reach the blastocyst stage in group culture.

2. Introduction

The merits of culturing embryos individually or in groups has been debated for almost 20 years. To date, contradictory reports have been published on the benefit of group culture over individual culture for human embryos (Ebner, et al. 2010, Rebollar-Lazaro and Matson 2010, Rijnders and Jansen 1999, Spyropoulou, et al. 1999, Tao, et al. 2013) whereas many papers have described the benefits of group culture for bovine (Donnay, et al. 1997, Goovaerts, et al. 2009, O'Doherty, et al. 1997, Wydooghe, et al. 2014), mouse (Canseco, et al. 1992, Lane and Gardner 1992, Paria and Dey 1990, Salahuddin, et al. 1995), pig (Stokes, et al. 2005) and cat (Spindler and Wildt 2002) embryos. The main reason for this beneficial effect of group culture on animal embryo development has been ascribed to the secretion of autocrine factors by preimplantation embryos that act upon the embryo itself or on neighbouring embryos (O'Neill 2008, Paria and Dey 1990). In contrast, proponents of individual culture of human embryos argue that, regardless of the production of these embryotrophic factors, embryos in groups may be exposed to negative effects of dying or delayed embryos cultured in the same drop (Reed, et al. 2011). Additionally, another practical reason in favour of individual culture is the ability to

follow morphological changes and development rates in individual embryos, which is of utmost importance in human IVF attempts.

Recently, new culture devices have been designed for human embryos that allow the benefits of both group and individual culture approaches to be combined. One such device is the Corral® dish (Sun IVF, Guelph, Canada), which consists of two central wells, each divided into 4 quadrants and 8 outer wells. The quadrant design allows the free passage of medium amongst the 4 quadrants, but the gaps between the quadrants are smaller than the diameter of an embryo so individual monitoring is possible. Another type of culture device is based on the Well-of-the-Well (WOW)-system which was developed initially for zona-free bovine embryos (Vajta, et al. 2000). In the bottom of a culture dish, microwells were created by fashioning small, round bottomed cylindrical wells and covering them with culture medium, to create in each well an optimum microenvironment for the embryo (Vajta, et al. 2000). Over the past decade, WOW dishes have been applied successfully to human IVF in combination with time-lapse cinematography. Two different models have been employed, the Embryo Slide® dish which allows the culture of individual embryos in microwells (Embryoscope) and the Primo Vision® dish (Cryo Innovation and Vitrolife) and Eeva® dish (Auxogyn) both of which employ the WOW principle to permit group culture of embryos, each held in its own microwell while sharing the same drop of culture medium.

The aim of this study was to examine the effects of group culture in more detail using bovine embryos. Bovine embryos are very sensitive to suboptimal culture conditions and, in general, they develop less well in individual culture (Goovaerts, et al. 2012). We compared the developmental outcome for bovine embryos cultured in these systems, with classical group culture (positive control: used routinely in bovine embryo culture (Fischer-Brown, et al. 2002, Goovaerts, et al. 2009, Sagirkaya, et al. 2007, Wydooghe, et al. 2014) and individual culture (negative control). Furthermore, by using Primo Vision® dishes we could investigate the influence of the developmental stage of the grouped embryos on the final developmental fate of any particular embryo cultured in a group compared to that of embryos cultured individually.

3. Materials and methods

3.1 Media and reagents

Basic Eagle's Medium amino acids, minimal essential medium non-essential amino acids (100 ×), TCM-199 medium, kanamycin and gentamycin were purchased from Life Technologies Europe

(Ghent, Belgium) and all other components were obtained from Sigma (Schnelldorf, Germany). All the media were filter-sterilized using a 0.22 µm filter (Pall Corporation, Ann Arbor, MI, USA) before use.

3.2 *In vitro* embryo production

Bovine embryos were produced by routine *in vitro* methods described previously by Wydooghe et al. (2014). Briefly, cow ovaries were collected from a local slaughterhouse and processed within 2 h. The ovaries were washed 3 times in warm physiological saline supplemented with kanamycin (25 mg/ml). The cumulus-oocyte complexes were aspirated from antral follicles between 4 and 8 mm diameter using an 18-gauge needle attached to a 10 ml syringe. Oocytes with uniformly granulated cytoplasm and surrounded by more than 3 compact layers of cumulus cells were selected and these were matured in groups of 60 in 500 µl modified bicarbonate-buffered TCM-199 supplemented with 20 ng/ml epidermal growth factor (EGF) and 50 µg/ml gentamicin in 5% CO_2 in air for 22 h at 38.5 °C. Frozen–thawed bull spermatozoa were passed over a Percoll gradient (45 and 90%; GE Healthcare Biosciences, Uppsala, Sweden) and then washed. The final concentration of 1×10^6 sperm/ml was adjusted using IVF-Tyrode's Albumin-Pyruvate-Lactate (TALP), which consisted of bicarbonate buffered Tyrode's solution, supplemented with BSA (Sigma A8806; 6 mg/ml) and heparin (25 µg/ml). The matured oocytes were washed in 500 µl of this IVF–TALP and incubated with spermatozoa for 21 h. After fertilisation, surplus spermatozoa and cumulus cells were removed by vortexing and the inseminated oocytes were transferred to synthetic oviduct fluid supplemented with essential and non-essential amino acids (SOFaa) plus 0.4% BSA (Sigma A9647) and ITS (5 µg/ml Insulin + 5 µg/ml Transferrin + 5 ng/ml Selenium). The number of inseminated oocytes per group and the volume of culture medium they were incubated in, depended on the experimental set-up.

3.3 Experiment 1: Comparison of different group culture systems

A total of 1572 bovine oocytes (4 replicates) were randomly allocated to 4 experimental groups as detailed in Table 1. Two of the systems have been approved for use in human embryo culture (Primo Vision® dish and Corral® dish; Figures 1B & 1C) and the other two systems (group and individual culture; Figure 1A & 1D) had been tested previously in our laboratory (Wydooghe et al. 2014). For group culture, 4 drops of 50 µl were placed in one Petri dish and covered by 7.5 ml of mineral oil, whereas for individual culture per Petri dish 17 drops of 20 µl were covered by 7.5 ml of mineral oil. In each Primo Vision® dish, 1 drop of 30 µl medium was covered by 2.5 ml of mineral oil and in each Corral® dish, 2 drops of 120 µl culture medium were covered by 13 ml of mineral oil. It should be noted

that in this experimental set-up, the differences in embryo density (concerning both culture medium and oil overlay) are uncontrolled and may be a significant confounder in the interpretation of the data.

Table 1. Summary of the different culture systems used.

	Group	Primo Vision®	Corral®	Individual
Drop size (µl)	50	30	120	20
Embryos/drop	25	10	4	1
Embryo density	1:2	1:3	1:30	1:20
Inter-embryo distance *	1-500 µm	680 µm	4 000 µm	–

**Measured from centre-point to centre-point.*

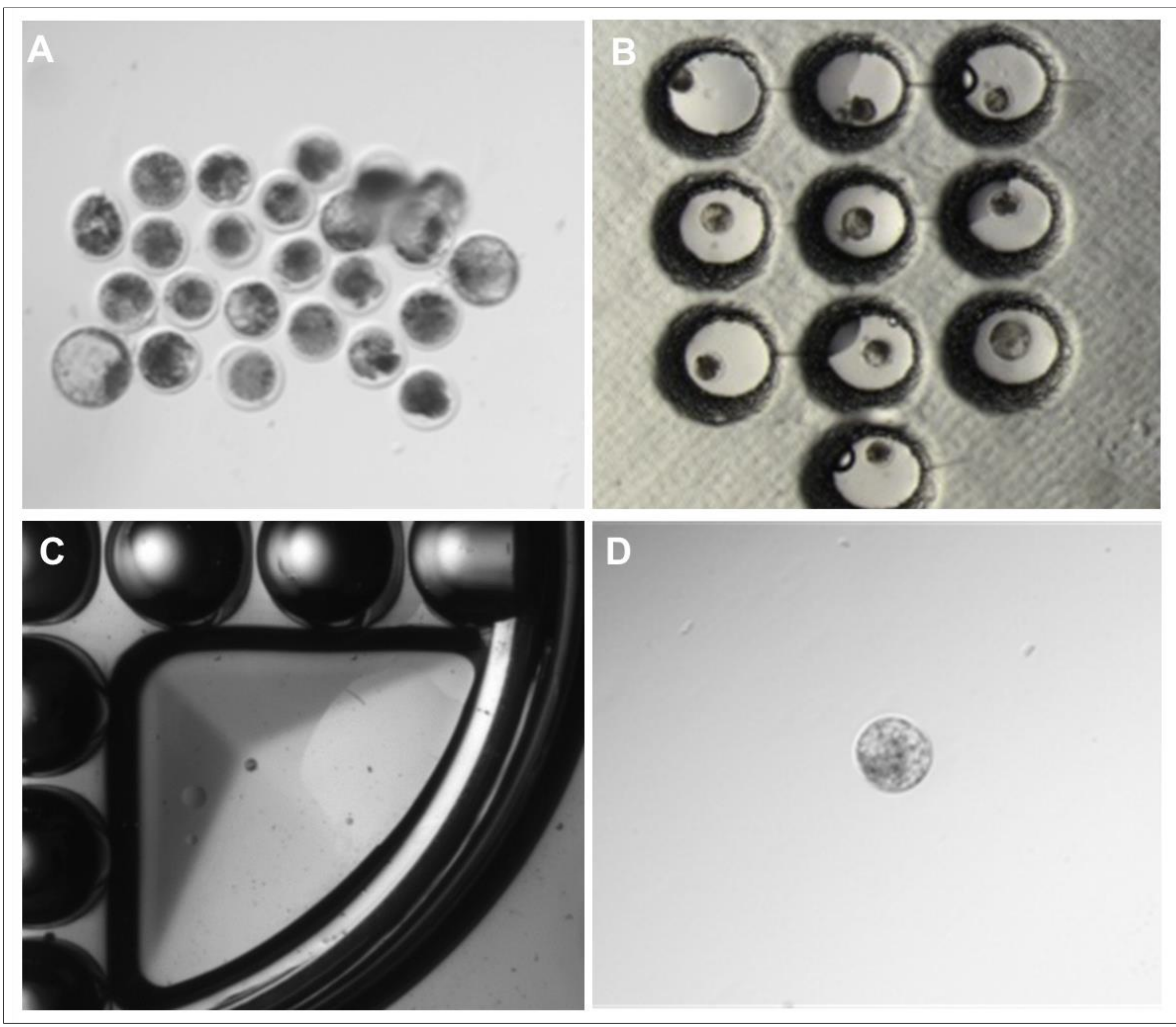

Figure 1. *Different culture systems investigated: A. Group culture: inseminated oocytes were cultured in groups of 25 in a 50 µl drop of culture medium covered with mineral oil. B. Group culture in Primo Vision® dishes: 10 inseminated oocytes were placed in the 10 microwells (one embryo per microwell)*

and covered by a single drop of 30 µl culture medium and mineral oil. C. Group culture in embryo Corral® dishes: one embryo was placed in each quadrant of the centre well, so that 4 inseminated oocytes were cultured as a group, divided by a raster in a drop of 120 µl culture medium covered by mineral oil. D. Individual culture: inseminated oocytes were cultured individually in 20 µl drops of medium. Seventeen drops were placed in one plate and covered by mineral oil.

3.4 Experiment 2: Influence of non-cleaved embryos in group culture

After fertilisation, all inseminated oocytes (n = 629, 5 replicates) were cultured in Primo Vision® dishes. During the course of the experiments the Primo Vision® dish was modified from that designed for 10 embryos to one designed for 9 embryos arranged in a 3 x 3 raster. In experiment 2, therefore, all inseminated oocytes were cultured in groups of 9, with each of them in one microwell, and immersed in 30 µl SOF medium. At 45 hours post insemination (hpi), embryo cleavage was assessed and 3 experimental groups were created in which; i) no additional handling was performed (CTRL), ii) non-cleaved embryos (NC) were removed (NC removed); iii) non-cleaved embryos were removed and the cleaved embryos were grouped together (ReGR).

3.5 Evaluation of embryo development

At 45 hpi embryo cleavage rate was evaluated as the percentage of inseminated oocytes that cleaved. The kinetics of cleavage was also assessed and embryos that had proceeded to the third cleavage division (5–8 cells) were classified as 'fast embryos' whereas those that had reached only the second cleavage division (2–4 cells) were categorized as 'slow embryos' (Vandaele, et al. 2007). The percentage of inseminated oocytes to reach the blastocyst stage of development was assessed at 168 and 192 hpi and at the latter time point the percentage of blastocysts that had hatched from their zona pellucida was also recorded.

3.6 Evaluation of embryo quality

At 192 hpi all the blastocysts were fixed in 2% paraformaldehyde (w/v) for 20 min and subsequently subjected to differential apoptotic staining to assess the quality of their morphological development (Wydooghe, et al. 2011b). This involved double immunofluorescent staining with firstly, CDX2, a transcription factor uniquely expressed in the trophectoderm cells (TE), secondly, with active caspase-3 which plays a central role in all apoptotic pathways. This staining was further combined with a Hoechst nuclear stain. This method allowed simultaneous evaluation of 3 important parameters of embryo quality; i) the total cell number (TCN), ii) the proportion of inner cell mass cells (ICM ratio) calculated by subtraction of the number of TE cells from the TCN, divided by the TCN and iii) the

percentage of apoptotic cells (apoptotic cell ratio, ACR). Additionally the absolute ICM-number (calculated as TCN-TE) and apoptotic cell number were also counted.

3.7 Comparative efficiency of group versus individual culture methods

Comparison of the embryos in Primo Vision® culture dishes with those cultured individually in Experiment 1, gave valuable insight into the group culture phenomenon. Retrospectively, therefore, blastocyst development at 192 hpi for those groups cultured in Primo Vision® dishes versus those cultured individually was related to whether the embryos had been 'slow' (2–4 cells) or 'fast' (5–8 cells) developers at 45 hpi, and what effect, if any, the number of neighbouring embryos may have had on the rates of blastocyst development of both 'fast' and 'slow' embryos. Similarly, did the stage of development of neighbouring embryos in Primo Vision® dishes improve or impair blastocyst development. Finally, the effect upon the embryos of being cultured in a 'standard' or a 'delayed' drop, were investigated, based upon the development stages of the 9 embryos in the group. The definition of 'standard' and 'delayed' drop in the Primo Vision® culture at the different time points was based on the embryo development in the control group (classical group culture) (Table 2).

Table 2. Definition of standard and delayed drops in Primo Vision® dishes based on the average percentage obtained in the classical group culture system.

Time post *in vitro* insemination	Embryo Stage	Reference value in classical group culture system		No. embryos in droplet which reached the same stage in a Primo Vision® dish	
				Delayed drop	Standard drop
45 hpi	Not cleaved	20.6%	=>	> 2 / 9	≤ 2 / 9
	Fast embryos	18.8%	=>	< 2 / 9	≥ 2 / 9
168 hpi	Blastocyst	23.1%	=>	< 2 / 9	≥ 2 / 9
192 hpi		34.3%	=>	< 3 / 9	≥ 3 / 9

3.8 Statistical analysis

To analyse the data on embryo development in both experiments, a binary logistic regression model of IBM SPSS Statistics 19 was used, with group and replicate as fixed effects. A Bonferroni correction was performed to correct for the multiple comparison. Significance level was set at a corrected P value < 0.05. Data concerning the embryo quality such as TCN, ICM ratio, ACR and absolute ICM-number and apoptotic cell number were analysed using a mixed model analysis of variance, with

group as the fixed effect and replicate as the random effect. Since the data concerning ACR in Experiment 1 were not normally distributed, a log transformation of the data was applied in order to obtain normally distributed (Kolmogorov-Smirnov test P value > 0.01) and homogeneous data (Levene Test P value > 0.05). Evaluation of the strengths and weaknesses of group culture was carried out retrospectively using the data from Experiment 1. All variables were tested simultaneously using a binary logistic regression model of IBM SPSS Statistics 19, with group and replicate as fixed effects. Differences where P < 0.05 were considered significant.

4. Results

4.1 Experiment 1: Comparison of different group culture systems

The embryo culture system did not affect the embryo cleavage rate at 45 hpi. Similarly, no differences were observed in the proportion of 'fast' (5–8 cell stage) and 'slow' (2–4 cell stage) embryos (Table 3). Blastocyst development at 168 hpi was not significantly different between the culture systems. At 192 hpi, however, a higher proportion of blastocysts were observed in Primo Vision® dishes compared to individually cultured embryos and those cultured in Corral® dishes. Blastocyst development at 192 hpi in Primo Vision® dishes was not different from that in the classical group culture system. No differences in hatching rate were observed between the culture systems investigated.

Table 3. Embryo development in the different culture systems

		Cleavage rate at 45 hpi (% ± SD)		Blastocyst yield (% ± SD)		
	n	Fast embryos	Slow embryos	168 hpi[A]	192 hpi[A]	Hatching rate[B]
Group	399	18.8 ± 1.96	60.9 ± 2.21	23.1 ± 2.11	34.3 ± 2.38[a]	19.7 ± 3.40
Primo Vision®	382	18.6 ± 1.99	65.2 ± 2.06	25.9 ± 2.24	39.0 ± 2.22[a,b]	13.4 ± 2.79
Corral®	384	18.5 ± 1.98	56.5 ± 2.53	17.7 ± 1.95	28.9 ± 2.31[a,c]	15.3 ± 3.42
Individual	407	18.7 ± 1.93	59.2 ± 2.44	20.1 ± 1.99	28.5 ± 2.24[a,c]	16.4 ± 3.44

[A] *Blastocyst yield is expressed as the percentage of blastocysts at 168 hpi and 192 hpi respectively out of the number of presumed zygotes.*
[B] *Hatching rates are expressed as the percentage of hatching or hatched blastocysts at 192 hpi compared with the total number of blastocysts.*
Data are expressed as mean ± SD, differences at P < 0.05 were considered significant and are marked with different superscript.

Embryo quality was evaluated by simultaneously measuring 3 parameters; i) the total cell number (TCN); ii) the ICM ratio as the number of cells of the inner cell mass compared to the TCN; iii) the apoptotic cell ratio (ACR). Higher TCNs were recorded in blastocysts derived from classical group culture and culture in Primo Vision® dishes compared to individual culture and culture in Corral® dishes. The ICM ratio was similar for all 4 groups, although the actual number of ICM cells was higher in embryos cultured in classical group culture or in Primo Vision® dishes, compared to Corral® dishes or individual culture (GR: 67.6 ± 1.93a; Primo Vision®: 62.8 ± 1.89a; Corral®: 50.7 ± 1.98b; IND: 50.4 ± 1.51b). The ACR was higher for all the test groups compared to classical group culture (Figure 2). However, the actual number of apoptotic cells was higher only in blastocysts cultured in Corral® dishes compared to classical group culture (GR: 7.6 ± 0.26^{ab}; Primo Vision®: 8.6 ± 0.32^{a}; Corral®: 8.9 ± 0.35^{ac} ; IND: 8.0 ± 0.24^{a}).

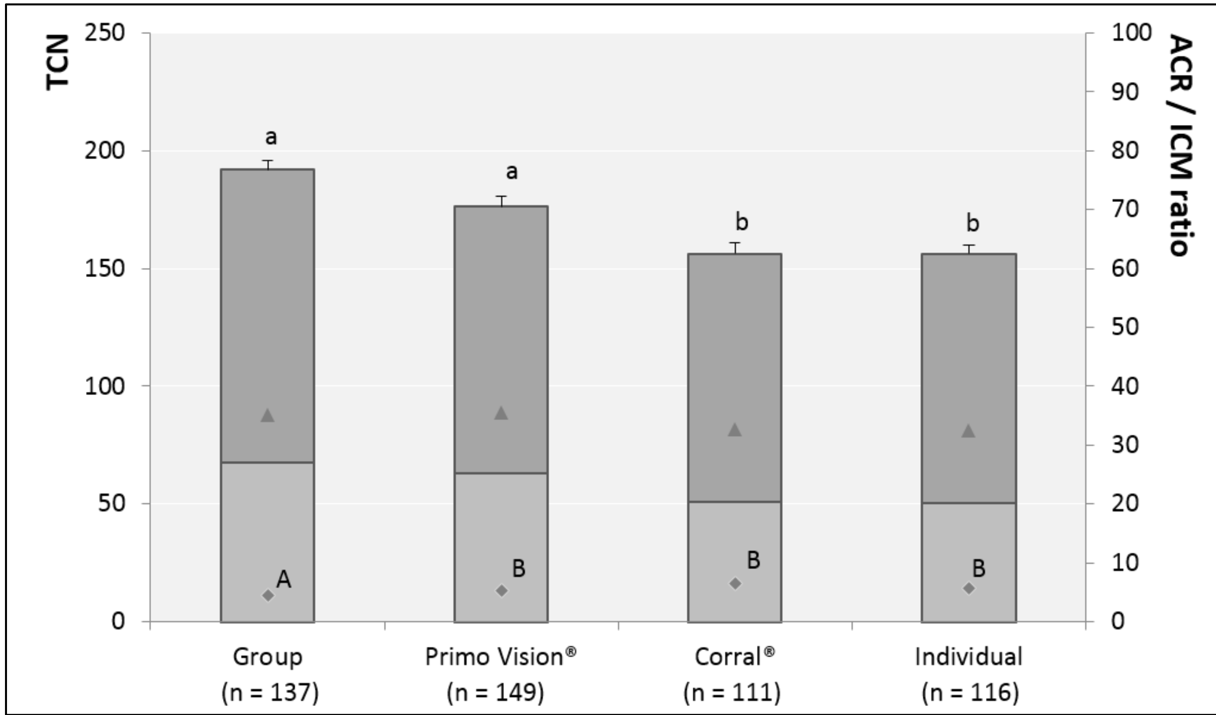

Figure 2. *Embryo quality in the different culture systems: Total cell number (TCN) as the sum of trophectoderm cells (TE; dark grey) and cells allocated to the ICM (calculated as TCN – TE) (light grey), ICM-ratio (▲) and apoptotic cell ratio (ACR; ◆) of blastocysts collected after 192 hpi and differential apoptotic staining. Embryos were cultured in classical group cultures, Primo Vision® dishes, Corral® dishes or as individuals; differences between the different culture systems that were significant (P < 0.05) are marked with a different superscript.*

4.2 Efficiency of group culture versus individual culture methods

In total, blastocyst development was compared for 245 ‘slow’ embryos in Primo Vision® dishes and 242 ‘slow’ embryos cultured individually; 75 ‘fast’ embryos cultured in Primo Vision® dishes and 77 ‘fast’ embryos cultured individually. The higher rate of blastocyst development at 192 hpi observed in Primo Vision® dishes compared to individual culture was due mainly to a significant increase in the development of ‘slow’ embryos to blastocyst stage in Primo Vision® dishes compared to individual culture (Table 4). No such increase in blastocyst development occurred for ‘fast’ embryos in Primo Vision® dishes compared to individual culture (Table 5).

Depending upon their position in the Primo Vision® dish, individual embryos could be accompanied by 3, 4, 5, 6 or 8 adjacent embryos, defined as ‘neighbours’ (Figure 3). The proportion of ‘slow’ embryos that reached blastocyst stage at 192 hpi, was higher for Primo Vision® dishes than for those cultured individually, especially if they were surrounded by 4 to 6 neighbours (Table 4 part 1). For fast embryos, however, the number of neighbours in Primo Vision® dishes did not affect the proportion of embryos that developed into blastocysts (Table 5 part 1).

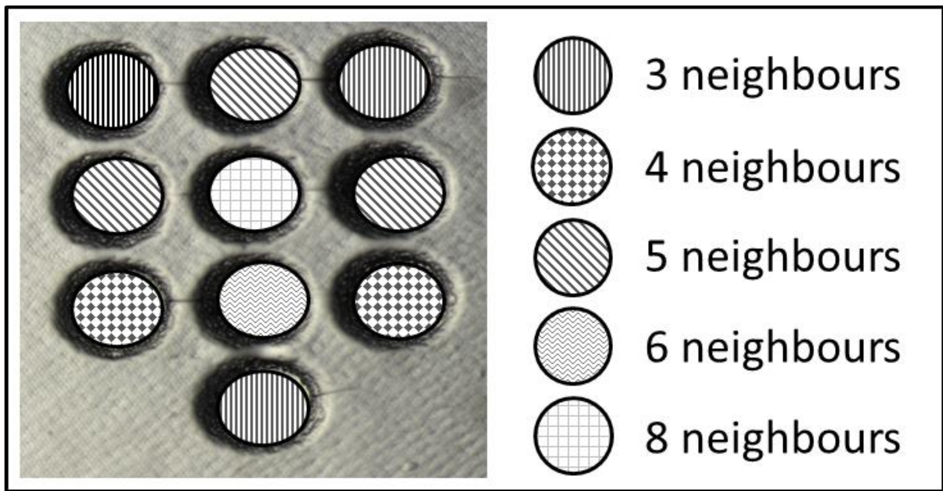

Figure 3. *Presentation of the number of neighbours in Primo Vision®: Depending on their position in the Primo Vision® dish, embryos could have 3, 4, 5, 6 or 8 neighbours (adjacent embryos).*

In addition to the analysis of the effect of the number of adjacent embryos, the effect of the developmental stage of the ‘neighbours’ at different time points was also examined. This involved looking retrospectively at the developmental stage of neighbouring embryos at 45 hpi and recording the percentage that were non-cleaved (unfertilized oocytes or arrested embryos) designated as neighbours with delayed development. We analysed if the percentage of neighbours with delayed development for embryos cultured in a Primo-vision® dish, (containing either 0-25%, 26-50%, 51-75%

and 76-100% non-cleaved or arrested embryos) affected blastocyst development at 192 hpi of both 'slow' and 'fast' embryos versus embryos cultured individually. Blastocyst development of 'slow' embryos was enhanced in Primo Vision® dishes compared to individual culture when < 25% of the neighbouring embryos were non-cleaved at 45 hpi. However, when more than 25% of neighbouring embryos were non-cleaved at 45 hpi, development of 'slow' embryos to blastocyst stage was not different from the rate of 'slow' embryos cultured individually (Table 4 part 2A). For 'fast' embryos, the proportion of neighbouring non-cleaved embryos did not reduce blastocyst development compared to individually cultured 'fast' embryos (Table 5, part 2A).

Using the same approach to analyse the influence of having adjoining embryos showing a standard rate of development (i.e. 'fast' cleaving embryos at 45 hpi and embryos reaching the blastocyst stage at 168 and 192 hpi) it transpired that when either 0–25 or 26–50% of the neighbours of 'slow' embryos were 'fast' cleavers at 45 hpi, a higher rate of blastocyst development by 'slow' embryos compared to their individually cultured counterparts was observed (data not shown). On the contrary, for 'fast' embryos the proportion of 'fast' cleaving neighbours did not influence the blastocyst development at 192 hpi compared to individual culture (data not shown). The proportion of neighbours of 'slow' embryos that reached the blastocyst stage at 168 hpi and 192 hpi (Table IV 4 part 2.B and 2.C) is likely to be less important; all the categories scored better than individual culture, except for the group of 26–50% of the neighbours developing into a blastocyst at 192 hpi. For 'fast' embryos, on the other hand, no differences were observed between the individually cultured 'fast' embryos versus those cultured in groups however a higher number of embryos is needed to confirm these observations (Table 5 part 2B and 2C).

Finally, examination of the effect of being in a so-called 'standard' or a 'delayed' drop showed that the number of non-cleaved embryos in Primo Vision® dishes was not associated with a reduced rate of blastocyst development in group culture; this was the case for both 'slow' and 'fast' embryos. When a particular embryo was a 'slow' embryo, it benefited from group culture in Primo Vision® dishes, as reflected by a higher rate of blastocyst development at 192 hpi compared to individually cultured 'slow' embryos. This was not dependent on the number of non-cleaved embryos at 45 hpi (Table 4 part 3D). By contrast, 'fast' embryos did not show a higher rate of blastocyst development at 192 hpi following group versus individual culture, regardless of whether the embryo resided in a drop with two or more non-cleaved embryos (Table 5, part 3D). The number of 'fast' embryos in the drop at 45 hpi, did not influence the rate of blastocyst development of either 'slow' or 'fast' embryos in group culture compared to their individually cultured counterparts (data not shown). 'Slow' embryos benefited from the vicinity of more than 2 blastocysts at 168 hpi and this was even more pronounced

Table 4. Influence of the number of neighbours and developmental stage of the neighbours and the grouped embryos on the blastocyst development of SLOW EMBRYOS (2-4 cells at 45 hpi) compared to their individually cultured counterparts.

	n	Blastocyst rate 192 hpi	p-value	Odds ratio + 95% CI
Individual	242	28.5		
Primovision	245	42.4 *	0.001	
1. Influence of number of neighbours				
3 neighbours	78	37.2	0.150	
4 neighbours	50	50.0 *	0.004	
5 neighbours	69	39.1	0.094	
6 neighbours	26	57.7 *	0.004	
8 neighbours	22	36.4	0.440	
2. Influence of the developmental stage of the neighbours				
A. Neighbours not cleaved at 45 hpi			**0.041**	
0 – 25%	193	43.0 *	0.002	
26 – 50%	47	38.3	0.183	
51 – 75%	3	33.3	0.855	
76 – 100%	2	100.0	0.999	
B. Neighbours blastocyst at 168 hpi			**0.003**	
0 – 25%	143	38.5 *	0.044	
26 – 50%	69	43.5 *	0.020	
51 – 75%	24	58.3 *	0.004	
76 – 100%	11	63.6 *	0.021	
C. Neighbours blastocyst at 192 hpi			**0.002**	
0 – 25%	83	41.0 *	0.036	
26 – 50%	90	34.4	0.296	
51 – 75%	50	54.0 *	0.001	
76 – 100%	22	54.5 *	0.015	
3. Influence of the developmental stage of the grouped embryos				
D. Not cleaved at 45 hpi in the drop			**0.006**	
Delayed drop	38	47.4 *	0.022	
Standard drop	206	41.3 *	0.005	
E. Blastocysts at 168 hpi in the drop			**0.001**	
Delayed drop	85	34.1	0.333	
Standard drop	160	46.9 *	0.000	
F. Blastocyst at 192 hpi in the drop			**0.000**	
Delayed drop	81	30.9	0.687	
Standard drop	164	48.2 *	0.000	

Table 5. Influence of the number of neighbours and developmental stage of the neighbours and the grouped embryos on the blastocyst development of FAST EMBRYOS (5-8 cells at 45 hpi) compared to their individually cultured counterparts.

	n	Blastocyst rate 192 hpi	p-value	Odds ratio + 95% CI
Individual	77	59.7		
Primovision	75	52.0	0.337	
1. Influence of number of neighbours			**0.257**	
3 neighbours	26	65.4	…	
4 neighbours	9	33.3	…	
5 neighbours	26	53.8	…	
6 neighbours	3	66.7	…	
8 neighbours	11	27.3	…	
2. Influence of the developmental stage of the neighbors				
A. Neighbours not cleaved at 45 hpi			**0.872**	
0 – 25%	59	59.7	…	
26 – 50%	14	52.5	…	
51 – 75%	2	0.0	…	
76 – 100%	0			
B. Neighbours blastocyst at 168 hpi			**0.123**	
0 – 25%	44	40.9	…	
26 – 50%	20	55.0	…	
51 – 75%	9	88.9	…	
76 – 100%	2	100	…	
C. Neighbours blastocyst at 192 hpi			**0.191**	
0 – 25%	27	40.7	…	
26 – 50%	24	45.8	…	
51 – 75%	19	68.4	…	
76 – 100%	5	80.0	…	
3. Influence of the developmental stage of the grouped embryos				
D. Not cleaved at 45 hpi in the drop			**0.567**	
Delayed drop	13	46.2	…	
Standard drop	62	53.2	…	
E. Blastocyst at 168 hpi in the drop			**0.390**	
Delayed drop	29	44.8	…	
Standard drop	46	56.5	…	
F. Blastocyst at 192 hpi in the drop			**0.039**	
Delayed drop	27	33.3 *	0.021	
Standard drop	48	62.5	0.759	

Odds ratio axis: 0.0, **1.0**, 2.0, 4.0, 6.0, 50.0

at 192 hpi (Table 4 part 3E and 3F). For 'fast' embryos however, if < 3 of the embryos in the group had developed into a blastocyst at 192 hpi, the chance of that particular embryo becoming a blastocyst compared to individually cultured 'fast' embryos was significantly reduced (Table 5 part 3F).

4.3 Experiment 2: Effect of non-cleaved embryos in group culture.

Removal of non-cleaved embryos from the culture, with or without regrouping the cleaved embryos afterwards, did not influence embryo cleavage at 45 hpi. Similarly, no differences were observed in the proportions of 'fast' (5–8 cells) and 'slow' embryos (2–4 cells; Table 6). Blastocyst development at 168 hpi and 192 hpi was likewise not affected. However, a higher hatching rate was observed at 192 hpi when the cleaved embryos were regrouped after removal of the non-cleaved embryos compared to the control. No differences in blastocyst quality could be observed between the groups in terms of TCN, ICM-ratio or ACR (Figure 4), absolute ICM-number (CTRL: 67.1 ± 2.61; NC Removed: 56.0 ± 2.57; ReGR: 66.7 ± 2.88) and apoptotic cell number (CTRL: 6.6 ± 0.42; NC removed: 6.8 ± 0.41; ReGr: 7.2 ± 0.45).

Table 6. Effect of removal of non-cleaved embryos or regrouping of cleaved embryos on embryo development in Primo Vision® dishes

		Cleavage rate at 45 hpi (% ± SD)		Blastocyst yield (% ± SD)		
Primo Vision®	n	Fast embryos	Slow embryos	168 hpi[A]	192 hpi[A]	Hatching rate[B]
CTRL	225	20.0 ± 2.67	60.0 ± 3.27	18.7 ± 2.60	31.6 ± 3.10	11.3 ± 3.76[a]
NC-Removed	225	16.0 ± 2.44	60.4 ± 3.26	15.1 ± 2.39	29.3 ± 3.03	9.1 ± 3.54[a]
ReGR	179	18.4 ± 2.90	63.7 ± 3.59	20.1 ± 2.99	29.6 ± 3.41	30.2 ± 6.31[b]

[A] Blastocyst yield is expressed as the percentage of blastocysts at 168 hpi and 192 hpi respectively out of the number of presumed zygotes.
[B] Hatching rates are expressed as the percentage of hatching or hatched blastocysts at 192 hpi compared with the total number of blastocysts.
Data are expressed as mean ± SD, differences at P < 0.05 were considered significant and are marked with different superscript.
CTRL = control; NC-Removed = removal of non-cleaved embryos; ReGr = removal of non-cleaved embryos with subsequent regrouping of the cleaved ones.

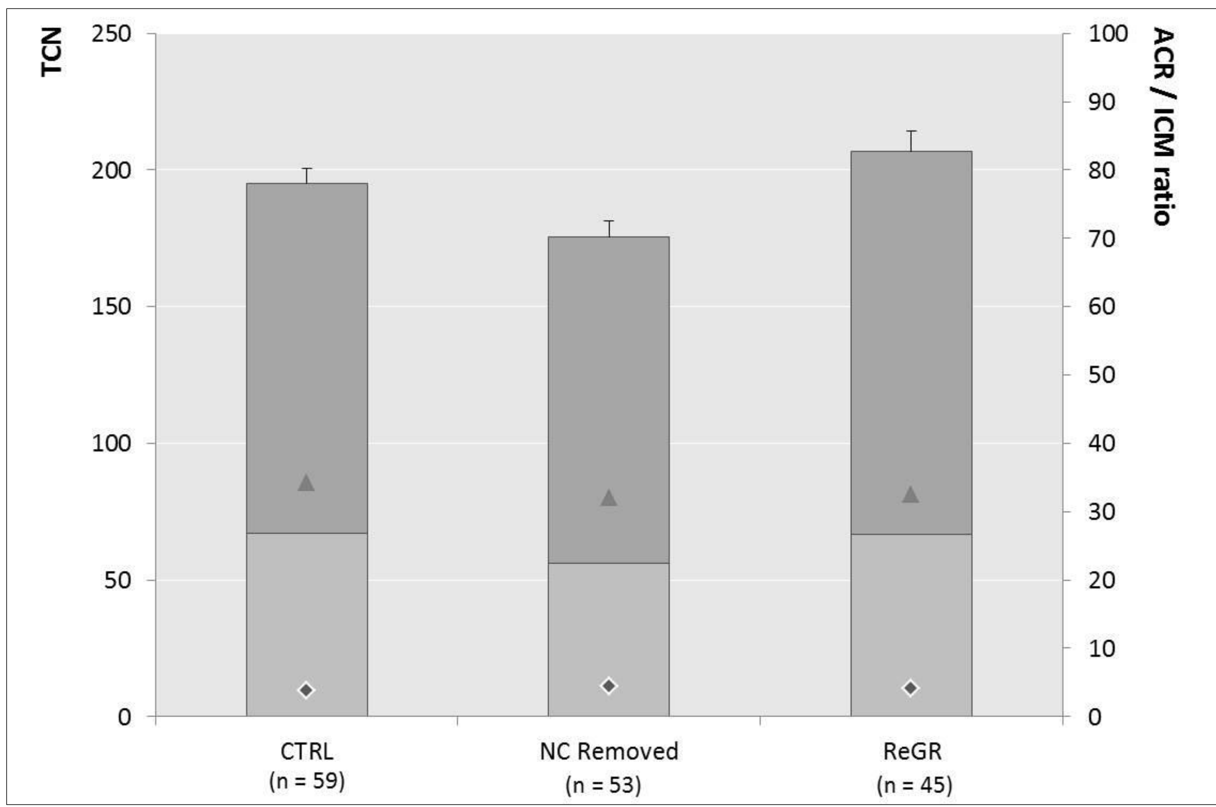

Figure 4. *Effect of removal of non-cleaved embryos or regrouping of cleaved embryos on embryo quality in Primo Vision® dishes: Total cell number (TCN) as the sum of trophectoderm cells (TE; dark grey) and cells allocated to the ICM (calculated as TCN - TE) (light grey), ICM-ratio (▲) and apoptotic cell ratio (ACR; ◆) of blastocysts collected at 192 hpi after differential apoptotic staining. Embryos were cultured in Primo Vision® dishes (CTRL), with removal of the non-cleaved embryos (NC Removed) and subsequent regrouping (ReGR).*

5. Discussion

In this study, two commercially available group culture systems, Corral® dishes and Primo Vision® dishes were compared, using bovine embryos and following the development of individual embryos. The results clearly showed that Primo Vision® dishes were advantageous compared to culture in Corral® dishes or culture of individual embryos in terms of both the development and quality of the embryos. Moreover, embryo development in Primo Vision® dishes was not different from that in a classical group culture system, thereby allowing the conclusion that Primo Vision® dishes combine the benefits of both group and individual culture of mammalian embryos.

There are several possible explanations for the improved embryo development in Primo Vision® as compared to Corral® culture dishes. First is the higher density of embryos in the Primo Vision® dishes, as shown in Table 1. For bovine and mouse embryos an embryo density of one embryo per 2 µl medium has been described as the optimum density to maximize the effects of autocrine

factors (Kato and Tsunoda 1994, Palasz and Thundathil 1998). Second, the smaller inter-embryo distance in Primo Vision® versus Corral® dishes is advantageous (Table 1). Gopichandran and Leese (2006) described an inter-embryo distance of 165 µm as being optimal for blastocyst formation of bovine embryos. Third, the microwells in the Primo Vision® dish contribute to more efficient autocrine communication between embryos. Group culture with individual follow-up in microwells was shown to significantly improve the developmental potential of such group-cultured embryos (Hoelker, et al. 2009, Vajta, et al. 2000). This improvement was not strictly related to the dish used since individual culture of bovine embryos in Primo Vision® dishes at a rate of 1 embryo per 30 µl medium did not result in blastocyst development (data not shown).

It is generally accepted that WOW culture provides a good compromise between the controversial needs of large volume (nutrition and dilution of toxic components) and small volume (concentration of autocrine factors) of culture medium. In a recent study, which used a mathematical model to calculate the concentration of secreted materials surrounding the embryo, Matsuura (2014) demonstrated that, when embryos are cultured in microwells, a higher concentration of secreted macromolecules (autocrine factors) and better diffusion of detrimental small molecules such as ammonia, can be expected compared to the situation in conventional culture systems. In microwells the direction of such diffusion is restricted to the vertical dimension, whereas in conventional systems diffusion is facilitated both vertically and horizontally a reduced concentration of autocrine factors around the embryos.

Although group culture is used extensively for mammalian embryos, there are no data available on the possibly negative effects of arrested or delayed embryos being cultured in the same drop as a normal embryo. Nor is there any evidence of which embryos benefit most from the group culture effect. In the present study however, it was possible to demonstrate that blastocyst development in Primo Vision® dishes is not different from classical group culture, but is significantly better than individual culture. This beneficial outcome resulted mainly from a higher rate of blastocyst development from 'slow' embryos which did much better in the Primo Vision® dish than in individual culture. Concern could arise due to more low-grade embryos developing to the blastocyst stage thereby implying that such blastocysts could be of lower quality. Since embryo development *in vitro* is slower than that *in vivo* (Van Soom, et al. 1997), 'fast' developing embryos have, historically, been preferred to 'slow' embryos. However, recent data from mice surprisingly cast doubt on this hypothesis. Namely 'slow' embryos are more *in vivo*-like with respect to genomic imprinting and expression of metabolic markers. 'Fast' embryos, on the other hand, show a loss of genomic imprinting,

perhaps because the kinetics of high cleavage rates leaves insufficient time to ensure the correct passage of epigenetic information (Velker, et al. 2012).

When studying the influence of the 'neighbours', it appears that the number of these does not hamper an embryo's potential to reach the blastocyst stage. This is in agreement with the previous study of Stokes *et al.* (2005) who could not demonstrate a significant effect on blastocyst development of a porcine embryo's position in a 4 x 4 grid. More importantly, it appears to be the developmental stage of the neighbours, and more specifically their development after 45 hpi, which positively affects the outcome of a particular embryo. A markedly higher percentage of 'slow' embryos had developed into blastocysts by 192 hpi if they had been surrounded by many embryos that had also developed into blastocysts at 168 or 192 hpi, when compared to 'slow' embryos cultured individually. This supportive effect of group culture is presumably related to the production of autocrine factors although the identity of most of these autocrine embryonic factors remains unknown.

For some growth factors, such as basic fibroblast growth factor (bFGF), insulin-like growth factor 1 and 2 (IGF I and II), interferon, platelet-derived growth factor alfa and transforming growth factor beta (TGF-β), it has been demonstrated that the appropriate mRNA is expressed in bovine preimplantation embryos (Watson *et al.* 1992). However, no studies have yet confirmed the presence of these growth factor proteins or of other potential autocrine factors, in culture medium conditioned by bovine embryos. To date only stress-related proteins have been identified (Wydooghe, et al. 2011a). In contrast, some secreted factors have already been identified for cultured human embryos and these can be used as biomarkers for embryo selection. The phospholipid, Platelet Activation Factor (PAF), was the first molecule identified in human embryo conditioned medium and it has now also been demonstrated in the culture medium of murine, sheep, rabbit and hamster embryos where it stimulates embryo metabolism, cell-cycle progression and embryo viability (Reviewed by O'Neill 2005). Furthermore, the level of PAF produced by human preimplantation embryos in culture medium is positively correlated to pregnancy rate in women (Roudebush, et al. 2002). Other proteins secreted by human embryos into the culture medium include soluble human leucocyte antigen-G (sHLA-G) (Noci, et al. 2005, Sher, et al. 2005); and ubiquitin (Katz-Jaffe, et al. 2006), the latter factor being correlated with on-going blastocyst development. Ubiquitin is a component of the ubiquitin-dependent proteasome system which is involved in a number of physiological processes in somatic cells, including transcription (reviewed by Geng, et al. 2012) and apoptosis (reviewed by Jesenberger and Jentsch 2002). Further research is needed to determine its importance as an autocrine factor, as well as to identify other autocrine factors in embryo culture medium.

In this study we showed that bovine embryos that had not cleaved by 45 hpi did not negatively affect the potential of embryos in the same group to become blastocysts at 192 hpi, irrespective of whether the embryo in question was a ‘fast’ or a ‘slow’ one. Furthermore, removing the non-cleaved embryos did not increase blastocyst development nor blastocyst quality; although regrouping the cleaved embryos after uncleaved embryo removal was able to increase their hatching rate. Neira *et al.* (2010) have shown that some of the growth factors of which Watson *et al*. (1992) identified the mRNA in bovine embryos, can facilitate the hatching of bovine blastocysts when added to the culture medium (e.g. IGF-I, bFGF, TGF-β).

Care should be taken when ‘fast’ embryos are group cultured and only a few of the surrounding embryos reach the blastocyst stage since this could compromise blastocyst development compared to individually cultured ‘fast’ embryos. ‘Fast’ developing embryos may be more sensitive to the negative effects of metabolic by-products such as ammonia or lactate or to depletion of necessary substrates by other embryos (Reed, et al. 2011). Our present findings indicate that removing ‘fast’ embryos from a Primo Vision® dish for subsequent culture individually may be beneficial whereas ‘slow’ embryos should remain in the group culture to yield a bigger number of blastocysts.

In summary, our results demonstrated that culturing bovine embryos in small groups in Primo Vision® dishes provides an excellent system that combines the benefits of group and individual culture, leads to a similar rate of embryo development compared to classical group culture and gives better embryo development and quality than individual culture or culture in Corral® dishes. When more than one third of neighbouring embryos developed into blastocysts at 192 hpi, ‘slow’ embryos had a higher chance of becoming a blastocyst in comparison to individual culture (48.2%; OR: 2.3). Whereas, when < 30% of the neighbouring embryos reached the blastocysts stage, blastulation of ‘fast’ embryos was less efficient than in individual culture (33.3%; OR: 0.3). The study also showed that the net outcome of group culture of bovine embryos in Primo Vision® dishes is advantageous compared to individual culture, giving a higher blastocyst development rate and improved blastocyst quality. Further research is needed to extrapolate these observations to human embryos.

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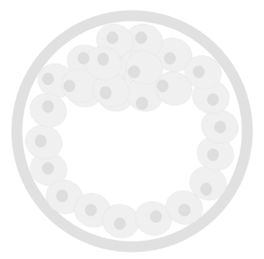


CHAPTER 6

SECRETOME ANALYSIS OF EMBRYO CONDITIONED MEDIUM

1. Abstract

Autocrine embryotropins are responsible for improved embryo development in group culture as compared to individual culture, but their identity remains largely unknown. Herein, we profiled proteins secreted by *in vitro* produced bovine preimplantation embryos. Albumin-free culture medium droplets, individually conditioned by excellent, good and poor quality embryos, were subjected to tandem mass spectrometric analysis. In medium derived from good embryos and excellent embryos, 22 and 17 proteins were identified respectively, that were absent in the blank medium or in the secretions of poor embryos. Amongst those, Cathepsin-L2 and SPARC were secreted by both good and excellent embryos; whereas alpha-S2-casein and pantetheinase were secreted by excellent embryos only. Cathepsin-L2 was further investigated for its embryotrophic properties. To this end, presumed zygotes were cultured individually in Synthetic Oviduct Fluid with or without 100 ng/ml Cathepsin-L. Cathepsin-L was (1) progressing the first cell divisions, (2) improving blastocyst development (31.0 ± 2.91. vs 22.9 ± 2.64) and (3) blastocyst quality, in terms of higher inner cell mass and lower apoptotic cell ratio, compared to individual embryo culture in the absence of Cathepsin-L. Taken together, we identified for the first time an embryo-derived protein, Cathepsin-L, playing critical role as embryotrophic factor during bovine preimplantation embryo development.

2. Introduction

Mammalian embryo culture has advanced significantly over the last decades, resulting in the use of assisted reproduction as infertility treatment for humans, cattle and horses (for review see, Galli, et al. 2014, Swain 2015). Further attempts to improve embryo culture systems have been mainly focused on enhancing media formulations (Wale and Gardner 2016). Culture media based on the salt concentrations of oviduct, such as Synthetic Oviduct Fluid, or SOF medium (Tervit, et al. 1972), are now used widely to culture ruminant embryos (Moreno, et al. 2015). Interestingly, animal studies have shown already three decades ago that culturing embryos in groups as opposed to individual culture significantly improves blastocyst cell number and viability (Donnay, et al. 1997, Lane and Gardner 1992, O'Doherty, et al. 1997, Paria and Dey 1990). In group culture, embryos are typically cultured in drops of 25-100 µL watery medium covered with mineral oil, at a ratio of 1 embryo per 1 or 2 µL of medium, whereas in individual culture, the embryo is cultured singly in a droplet of about 20 µL. The positive effect of group culture has been attributed to the secretion of autocrine factors (Paria and Dey 1990). These autocrine factors are signalling factors produced and released by embryos and acting upon the embryo itself or the neighbouring embryos (Gopichandran and Leese 2006, O'Neill 2008, O'Neill, et al. 2012, Stokes, et al. 2005). A wide range of biochemical messengers including proteins, lipids,

neurotransmitters, saccharides, and microRNAs can be exchanged between embryos cultured in group and might function as potential embryotrophic factors (for review see Wydooghe, et al. 2017). Proponents of individual culture, however, argue that embryos cultured in groups may be exposed to secreted detrimental factors released by poor quality embryos and furthermore they lose traceability that allows better embryo selection by continuous evaluation of embryo development (for review see Reed, et al. 2011). In this article, we want to respond to both arguments via identification of the autocrine factors secreted by embryos that permits supplementation of culture media to improve individual culture of embryos.

Recent advances in “omics” technologies have enabled the application of new molecular methods for the identification of these autocrine factors. The first studies focused on the transcriptome of preimplantation embryos by reverse transcription polymerase chain reaction (RT-PCR) (Watson, et al. 1992) whereas more recent attempts focused on the proteins expressed by preimplantation embryos, the so called ‘proteome’ (Demant, et al. 2015, Deutsch, et al. 2014). Lately, studies focused on the proteins secreted into the culture medium, or the so-called ‘secretome’. Secretomic analysis of culture media is becoming routine in human clinical practice as a non-invasive tool to predict successful embryo implantation (Cortezzi, et al. 2013, Dominguez, et al. 2015, Katz-Jaffe, et al. 2006, Sher, et al. 2005) or to select chromosomally normal embryos for transfer (McReynolds, et al. 2011). However, using a non-targeted approach, looking at full-scan mass spectrometry data to identify as many proteins as possible, was only used in a few studies in human (Combelles and Racowsky 2006, Cortezzi, et al. 2011, Foresta, et al. 2016, Katz-Jaffe, et al. 2009) and mouse (Beardsley, et al. 2010, Burch, et al. 2014). The main reason for this are the technical limitations in the detection of the low-abundant proteins of interest against the dominance of albumin, which is a common supplement of the culture media nowadays. In murine embryos, it was demonstrated that using albumin-free embryo culture medium led to a higher number of protein identifications compared to culture medium with albumin supplementation (Burch, et al. 2014). Another technical factor to consider is the fact that 50 to 70 % of human and bovine embryos cultured *in vitro* may arrest during development, whereas this is less than 10% in some mouse strains. Arrested or dying embryos may contain cells with a damaged cell membrane causing a passive leakage of proteins into the culture medium that might mask the actively secreted autocrine factors as well and lead to false conclusions.

Therefore, the objective of this study was to identify the proteins actively secreted or released in extracellular vesicles in albumin-free culture medium by *in vitro* produced bovine preimplantation embryos of good or excellent quality, using tandem mass-spectrometry. To this end, the embryos were cultured individually in defined embryo culture medium and additionally, the membrane integrity of

these *in vitro* produced embryos was checked in order to differentiate between good quality embryos and embryos of poor quality with a high percentage of membrane-damaged cells. Finally, Cathepsin-L, a protein identified as being actively secreted by good quality embryos, was added to the culture medium of individually cultured bovine embryos to verify its potential role as embryotrophic factor.

3. Materials and methods

Figure 1 presents a schematic overview of the workflow of this paper.

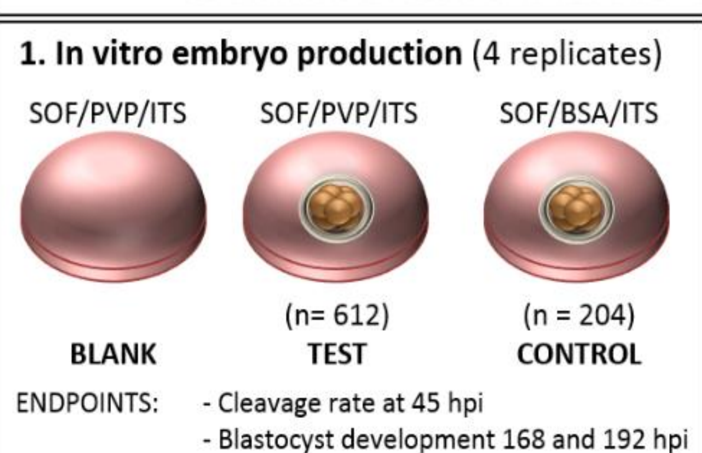
3.1 Media and reagents

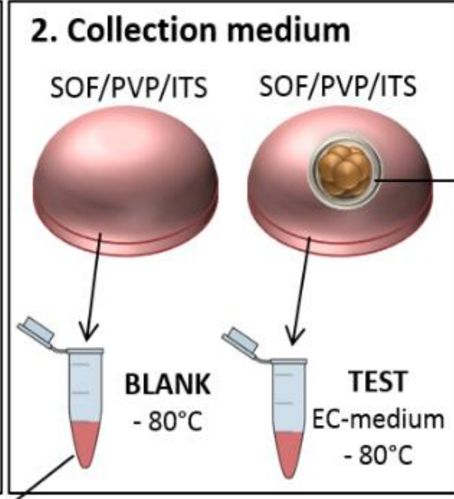
Basal medium eagle amino acids, minimal essential medium non-essential amino acids (100×), TCM-199-medium, kanamycin and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium) and all other components were obtained from Sigma-Aldrich (Diegem, Belgium). All media were filter-sterilized (0.22 µm Pall Corporation, Ann Arbor, MI, USA) before use.

3.2 *In vitro* embryo production in albumin-free culture medium and collection of embryo-conditioned media

Bovine embryos (n = 816, 4 replicates) were produced by previously-described routine *in vitro* methods (Wydooghe, et al. 2014a). Briefly, bovine ovaries were collected at the local slaughterhouse and processed within 2h. Ovaries were washed three times in warm physiological saline supplemented with kanamycin (25 mg/ml). Cumulus-oocyte complexes were collected and matured in groups of 60 in 500 µl modified bicarbonate-buffered TCM-199 supplemented with 20 ng/ml epidermal growth factor (EGF) and 50 µg/ml gentamicin for 22 h at 38.5°C in 5% CO_2 in air. Matured oocytes were washed in 500 µl IVF–TALP and incubated with Percoll selected frozen-thawed sperm (1×10^6 sp/ml) in IVF-TALP with heparin (25 µg/ml) for 21 hours. Next, excessive sperm and cumulus cells were removed by vortex and 204 presumed zygotes were transferred individually to drops of 20 µl of synthetic oviduct fluid supplemented with essential and non-essential amino acids (SOFaa), ITS (5 µg/ml Insulin + 5 µg/ml Transferrin + 5 ng/ml Selenium) and 4 mg/mL BSA (Sigma A9647) (SOF/ITS/BSA), serving as a positive control group. Furthermore, 612 presumed zygotes were cultured individually in 20 µl drops SOFaa supplemented with ITS and 0.1 mg/ml Polyvinylpyrrolidone (SOF/ITS/PVP). In each Petri dish 17 drops of 20 µl were covered by 7.5 ml of mineral oil and incubated at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2. An equivalent volume of blank SOF/PVP/ITS medium was incubated in the same conditions until 192 hpi. At 45 hpi, embryo cleavage rate was evaluated as the percentage of inseminated oocytes that cleaved. The percentage of inseminated oocytes reaching the blastocyst stage was assessed at 168

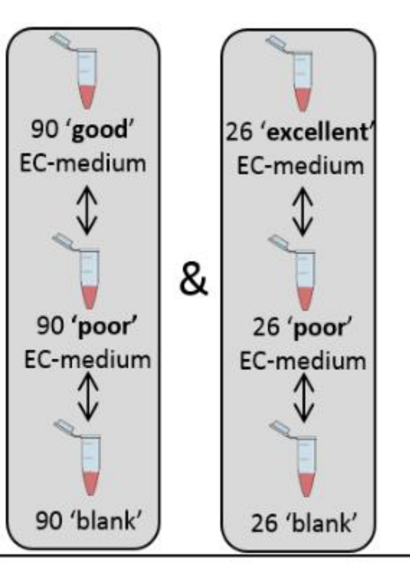
WORKFLOW: ANALYSIS OF DEFINED EMBRYO-CONDITIONED MEDIUM


1. In vitro embryo production (4 replicates)
SOF/PVP/ITS
SOF/PVP/ITS
SOF/BSA/ITS
(n= 612)
(n = 204)
BLANK
TEST
CONTROL
ENDPOINTS:
- Cleavage rate at 45 hpi
- Blastocyst development 168 and 192 hpi
- Hatching rate 192 hpi


2. Collection medium
SOF/PVP/ITS
SOF/PVP/ITS
BLANK
- 80°C
TEST
EC-medium
- 80°C

3. Determination TCN and MDC-ratio
Hoechst/Propidium iodide staining (n = 606):
: Membrane intact
: Membrane damaged
'Excellent' embryo: TCN > 64 cells, MDC-ratio = 0 %
'Good' embryo: TCN > 64 cells, MDC-ratio = 0.1 - 5 %
'Poor' embryo: TCN < 64 cells, MDC-ratio > 90 %

4. Protein identification


90 'good' EC-medium
90 'poor' EC-medium
90 'blank'
&
26 'excellent' EC-medium
26 'poor' EC-medium
26 'blank'
Pool EC- and blank medium (6 pools)
Protein precipitation
aceton (overnight – 20°C)
In solution trypsin digest
Mass spectrometric analysis
TripleTOF 5600 (Sciex)
Mascot Deamon version 2.4
Bovine database

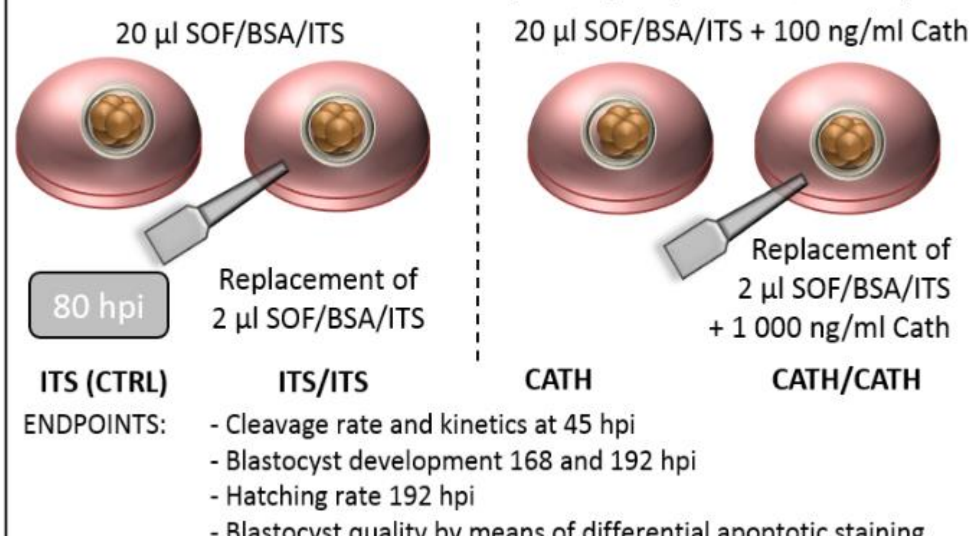

5. In vitro confirmation of Cathepsin-L (4 replicates, n= 988)
20 µl SOF/BSA/ITS
20 µl SOF/BSA/ITS + 100 ng/ml Cath
80 hpi
Replacement of 2 µl SOF/BSA/ITS
Replacement of 2 µl SOF/BSA/ITS + 1 000 ng/ml Cath
ITS (CTRL)
ITS/ITS
CATH
CATH/CATH
ENDPOINTS:
- Cleavage rate and kinetics at 45 hpi
- Blastocyst development 168 and 192 hpi
- Hatching rate 192 hpi
- Blastocyst quality by means of differential apoptotic staining

Figure 1. *Workflow: analysis of embryo-conditioned medium (EC-medium): (1) in vitro embryo production: individual culture of bovine embryos in drops of 20µl SOF/BSA/ITS, serving as control group to verify sufficient embryo development, and SOF/PVP/ITS. Drops of 20 µl SOF/PVP/ITS without embryo served as a blank. (2) Collection of medium: at 192 hpi, each drop of embryo-conditioned medium was collected separately, labelled and stored at – 80°C. A corresponding volume of blank medium was collected similarly. (3) Determination of total cell number (TCN) and membrane damaged cell-ratio (MDC-ratio): all embryos were subjected to a Hoechst/Propidium Iodide staining in order to differentiate between membrane intact and membrane damaged cells. Based on the results, embryos were categorized as 'excellent', 'good' or 'poor' quality. (4) Protein identification: the pooled embryo-conditioned medium (EC-medium) of 90 'good' and 90 'poor quality' embryos, and 90 blank drops was compared. Subsequently, 26 'excellent' and 26 'poor quality' embryos, and 26 blank drops were compared. (5) In vitro confirmation of Cathepsin-L: individual culture of bovine embryos in SOF/BSA/ITS, serving as a control, was compared to individual culture in SOF/BSA/ITS with 100 µg/ml Cathepsin-L. Additionally, at 80 hpi, for half of the embryos 10% (2 µl) of medium was replaced with or without 1000 µg/ml Cathepsin-L, in order to obtain 100 µg/ml in the final drop.*

and 192 hpi and at the latter time point, the percentage of hatching and hatched blastocysts was also recorded. After the culture period, all embryos cultured in SOF/PVP/ITS were removed from their droplet in a minimal amount of medium, using a micropipette under direct microscopic observation and subjected to a live fluorescent staining (see below). The remaining medium was withdrawn from each drop and collected separately in a LoBind Eppendorf tube (Eppendorf AG, Hamburg, Germany), labelled and stored at – 80°C until assayed. A corresponding volume of the incubated blank SOF/PVP/ITS medium was stored under similar conditions.

3.3 Determination of the ratio of membrane damaged cells

After 192 hpi, in total 606 embryos of the SOF/PVP/ITS group underwent a Hoechst/Propidium Iodide staining. This way, we were able to detect the total cell number (TCN) and the percentage of membrane-damaged cells (MDC-ratio) since Propidium Iodide cannot enter cells with intact cell membrane, thus membrane-damaged cells will stain pink instead of blue, as the membrane-intact cells do. Individual embryos were subjected, without fixation or permeabilisation treatment, to 18.6 µg/ml Propidium Iodide in PBS with 0.5% BSA (PBS/BSA) for 5 minutes at room temperature (RT). After a wash-step in PBS/BSA, the embryos were transferred to a 10 µg/ml Bisbenzimide (Hoechst 33342) solution in PBS/BSA for 10 minutes at RT. Subsequently, embryos were washed (PBS/BSA) and mounted individually on glass slides. The next day, samples were examined under an epifluorescence microscope (Leica DMR) using a A513804 filter cube (Excitation filter: BP 340-380; Dichromatic mirror: 400; Suppression filter: LP 425) allowing visualisation of both Hoechst and Propidium Iodide at the same time and assigned a label as excellent, good or poor quality (Figure 1).

3.4 Protein precipitation and in solution trypsin digest

After fluorescent staining, the embryo-conditioned medium of 26 ‘excellent’ embryos showing a normal embryo development (at least 64 cells at 192 hpi) and 0% membrane-damaged cells was pooled. The embryo-conditioned medium of 90 ‘good’ embryos with normal development showing only 0.1 to 5% membrane-damaged cells was pooled. In parallel, the embryo-conditioned medium of 26 and 90 embryos of ‘poor quality’ with arrested development (less than 64 cells at 192 hpi) and having at least 90% membrane-damaged cells was pooled in two separate Eppendorf’s. For the blank control, the same volume medium corresponding to respectively the volume of 26 and 90 drops of medium (in this case not conditioned by embryos), was pooled. The proteins in the 6 pools were precipitated by adding 9 volumes of ice-cold acetone and stored overnight at – 20°C. Afterwards, the pools were centrifuged at 20 800 g during 10 minutes at 4°C and acetone was discarded. Subsequently, they were dried in the Speedvac and solubilised in 20 µl 0.5M triethylammonium bicarbonate (TEABC), reduced with 2 µl 10mM Dithiotreitol and incubated at 60°C for 1h. After alkylation with 1 µl 200mM S-methylmethan-thiosulfate (MMTS, Fluka) (10 minutes at RT) the 6 pools were incubated overnight at 37°C, with trypsin (5 µg trypsin / 10 µl TEABC), 1mM $CaCl_2$ and 5% acetonitrile (ACN), dried in the Speedvac and stored at -20°C.

3.5 Protein identification by mass spectrometric analysis

Peptides were analysed on a TripleTOF 5600 (Sciex, Framingham, MA, USA) in a data dependent mode with dynamic accumulation automatically switching between MS (400 m/z-1250 m/z; accumulation time 0.25sec) and MS/MS (65-2000 m/z; accumulation time 0.025sec) when a threshold of 50cps was exceeded. These ions were excluded for further analysis for 30 seconds. Wiff files were converted to mgf format with Peakview 2.1 and data were searched against a bovine database (NCBI; 5984 entries) using Mascot Daemon version 2.4 (Matrix Science, London, UK). Peptide mass tolerance was set at 15ppm and fragment mass tolerance at 0.2Da. Two missed cleavages were allowed and identity threshold for peptides was set at p-value <0.01 and minimum 2 sequences per protein were requested.

First, a pool of 90 ‘good’ embryos with normal embryo development (TCN > 64 cells) and a MDC-ratio of only 0.1 to 5% was compared with a pool of 90 ‘poor quality’ embryos with arrested development (TCN < 64 cells) and a MDC-ratio of more than 90%. Simultaneously, the amount of medium corresponding to the volume of embryo-conditioned medium of 90 embryos was used as blank. Subsequently, a pool of 26 ‘excellent’ embryos with normal development with no membrane damaged cells at all, was compared with a pool of 26 ‘bad’ embryos with arrested development and

consisting of more than 90% of membrane damaged cells. The amount of medium corresponding to 26 drops was used as blank (Figure 1).

3.6 In *vitro* confirmation of relevance of Cathepsin-L in embryo development

This experiment was designed to confirm the autocrine effect of Cathepsin-L, one of the proteins identified in the embryo-conditioned medium of 'good' and 'excellent' embryos. Oocytes (n = 988; 4 replicates) were matured and fertilized *in vitro*, as previously described. After fertilisation, half of the presumed zygotes were cultured individually in drops of 20 µl SOF/BSA/ITS (ITS), and the other half were cultured in drops of 20 µl SOF/BSA/ITS supplemented with 100 ng/ml active human Cathepsin-L (Abcam, Cambridge, UK) (CATH) (Figure 1). The optimal concentration of Cathepsin-L was determined in preliminary dose-response experiments (data not shown). Since we did not know the turnover of Cathepsin-L in the current culture conditions, we decided to refresh 10% of the medium in order to add an additional amount of 100 ng/ml to the drop. At 80 hpi, for half of the CATH group 2 µl of medium was retrieved and replaced by 2 µl fresh SOF/BSA/ITS containing 1 000 ng/ml Cathepsin-L (CATH-CATH). For the other half of the CATH group no medium was replaced (CATH). Similarly, for half of the ITS group 2 µl of medium was refreshed with SOF/BSA/ITS at 80 hpi (ITS-ITS) and for the other half of the embryos, medium was not refreshed (ITS). The embryo development was evaluated at 45, 168 and 192 hpi as described earlier. Furthermore, the kinetics of cleavage was also assessed and embryos that had proceeded to the third cleavage division (5 - 8 cells) at 45 hpi were classified as 'fast embryos' whereas those that had reached only the second cleavage division (2 - 4 cells) were categorized as 'slow embryos' (Vandaele, et al. 2007). At 192 hpi, blastocysts were fixed in 2% paraformaldehyde (w/v) for 20 minutes and subsequently subjected to differential apoptotic staining to assess the quality of their morphological development (Wydooghe, et al. 2011). This method allowed simultaneous evaluation of three important parameters of embryo quality: (1) total cell number (TCN), (2) proportion of inner cell mass cells, as calculated by subtraction of the number of TE cells from the TCN (ICM ratio), divided by the TCN and (3) the percentage of apoptotic cells (apoptotic cell ratio (ACR)).

3.7 Statistical analysis

Data on embryo development were analysed using a binary logistic regression model of IBM SPSS Statistics 19, with test group and replicate as fixed effects. Data concerning the embryo quality such as TCN, ICM ratio and ACR were analysed using a mixed model ANOVA, with test group as the fixed effect and replicate as the random effect. Significance level was set at a P value < 0.05.

4. Results

4.1 Embryo development in albumin-free culture medium

Embryos cultured individually in SOF/PVP/ITS showed no differences in cleavage rate compared to individually cultured embryos in SOF/BSA/ITS (Table 1). At 168 hpi, blastocyst development in defined culture medium was inferior to SOF/BSA/ITS, but no differences were observed at 192 hpi (Table 1). The hatching rate however was significantly lower after individual culture in defined medium.

Table 1. Blastocyst development and hatching rates of embryos cultured individually in SOF ITS supplemented with either BSA (semi-defined) or PVP (defined).

Individual culture	Cleavage rate (%) 45 hpi	Blastocyst yield (%)		
		168 hpi[A]	192 hpi[A]	Hatching[B]
BSA	**75.0 ± 3.06**	**24.5 ± 3.04**	**33.5 ± 3.34**	**23.9 ± 5.21**
PVP	79.5 ± 1.63	12.8 ± 1.35 *	27.7 ± 1.81	3.5 ± 1.41 *

[A] *Blastocyst yield is expressed as the percentage of blastocysts at 168 hpi and 192 respectively out of the number of presumed zygotes.*
[B] *Hatching rates are expressed as the percentage of hatching or hatched blastocysts at 192 hpi compared with the total number of blastocysts.*
Data are expressed as mean ± SD, differences at $P < 0.05$ were considered significant and are marked with different superscript.

4.2 Determination of the ratio of membrane damaged cells

Using a Hoechst/Propidium Iodide staining, we were able to detect the TCN and MDC-ratio of all embryos cultured individually in defined medium (Figure 2). After determination of the TCN for each embryo, we categorized the embryos in two groups: normally developing embryos with a TCN of more than 64 cells (range: 64–313 cells; n=207) and arrested/delayed embryos with a TCN of less than 64 cells (range: 1-61 cells; n=399). In the first group, 90 embryos (43.5%) had a MDC-ratio of 0.01 to 5% and were classified as 'good' embryos, whereas 26 embryos (12.6%) showed no membrane-damaged cells at all, the so-called 'excellent' embryos. In the group with arrested or delayed development, only 60 embryos (15.3%) had a MDC-ratio of less than 5% and 54 embryos (13.5%) showed no membrane-damaged cells at all. Besides, 194 embryos (48.6%) showed a MDC-ratio of more than 50%, with most of them having even more than 90% of membrane-damaged cells (138 embryos; 34.5%).

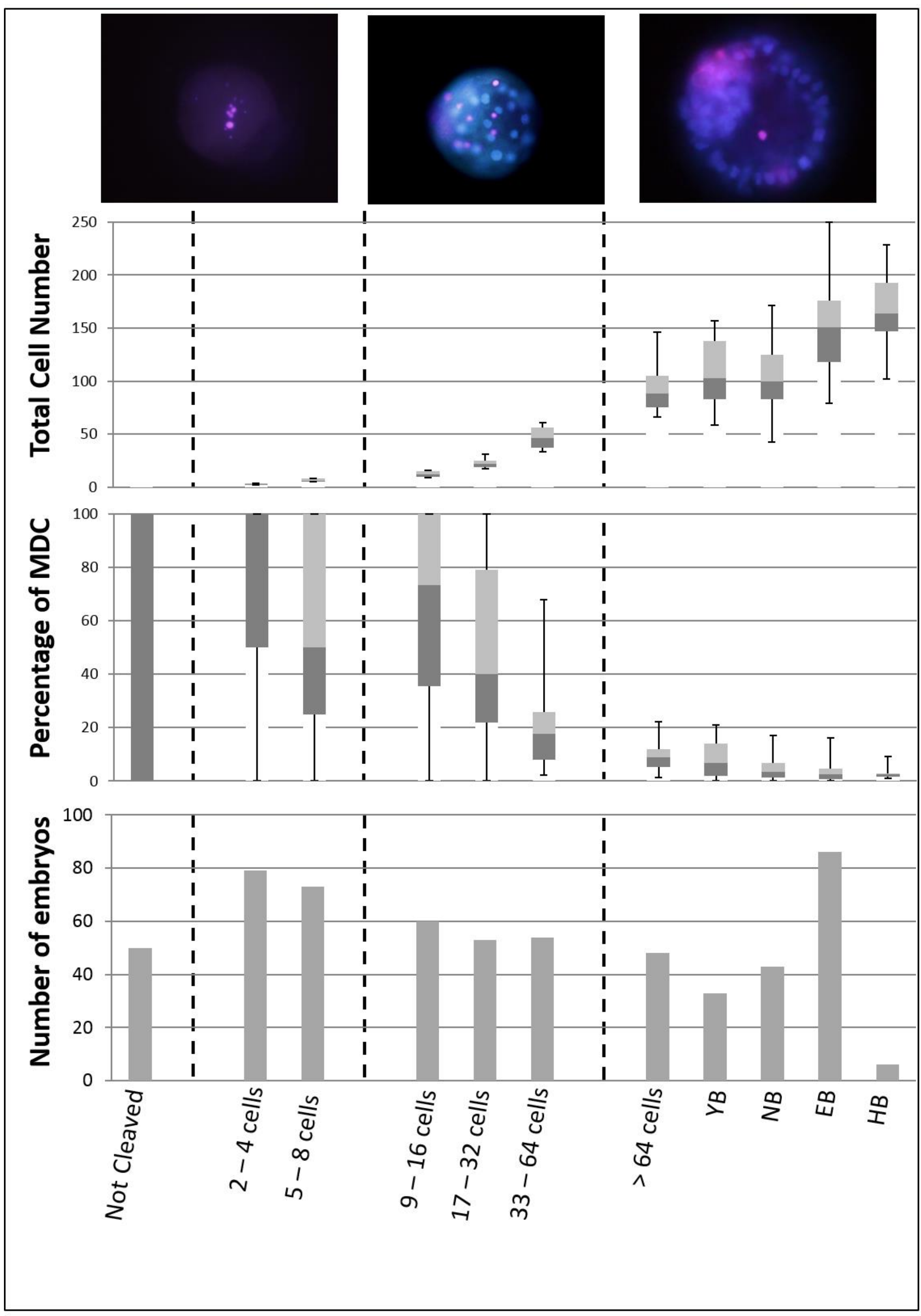


Figure 2. *Total cell number and occurrence of membrane damaged cells (MDC) in bovine embryos after 192 hours of culture. Embryos were cultured individually in drops of 20 µl SOF/PVP/ITS and at 192 hours post fertilisation (hpi) all embryos were subjected to a Hoechst/Propidium Iodide staining to assess the total cell number and the percentage of cells with damaged membrane. The box plots show the median*

values (transition dark grey –light grey), 25th and 75th percentile (boxes) and 5th and 95th percentile (whiskers). All embryos (n= 587) were collected from 4 replicates; the number of embryos belonging to a developmental stage is represented in the figure. Used abbreviations: MDC = Membrane damaged cells; YB = Young blastocyst; NB = Normal blastocyst; EB = Expanded blastocyst; HB = Hatching or Hatched blastocyst.

4.3 Protein identification by mass spectrometric analysis

In both samples of blank medium (corresponding to the volume of embryo-conditioned medium of 90 and 26 embryos, respectively), peptides from insulin, serum albumin and transferrin were identified (data not shown). In the pool of medium conditioned by 90 ‘good quality’ embryos (TCN > 64 cells; MDC-ratio: 0.1-5%), 22 proteins were identified. In the pool of 90 ‘poor quality’ embryos (TCN < 64 cells, MDC-ratio > 90%), 40 proteins were identified (Supplementary Table 1). Analogously, 17 proteins were detected in the pool of 26 embryos of ‘excellent quality’ (TCN > 64 cells and MDC- ratio = 0%) while 75 proteins were detected in the pool of medium conditioned by 26 embryos of ‘poor quality’ (TCN < 64 cells, and MDC-ratio > 90%) (Supplementary Table 2).

Although more proteins could be identified in the pool of medium conditioned by 26 embryos of ‘poor quality’, they confirmed the presence of 85% of the proteins identified in the pool of medium conditioned by 90 embryos of ‘poor quality’ (Table 2). Two proteins were present in the embryo-conditioned medium of both ‘good’ and ‘excellent’ embryos but not in the pool of medium conditioned by ‘poor quality’ embryos nor in the blank: Cathepsin-L2 (Q5E998|CATL2_BOVIN) and SPARC (P13213|SPRC_BOVIN). Furthermore, alpha-S2-casein (P02663|CASA2_BOVIN) and pantetheinase (Q58CQ9|VNN1_BOVIN) were only present in the embryo-conditioned medium of the 26 ‘excellent’ embryos and not in medium conditioned by ‘poor quality’ embryos. Proteins present in the pooled embryo-conditioned medium with 90 ‘good’ embryos but not in the medium of 26 ‘excellent’ embryos were not considered since they might have leaked from the small percentage of membrane-damaged cells.

Table 2. List of all proteins identified in albumin-free medium (SOF/PVP/ITS) conditioned by excellent[1], good[2] and poor[3] quality embryos and the blank sample.

Description	Accession	26 'excl'	26 'poor'	26 blank	90 'good'	90 'poor'	90 blank
Cathepsin-L2	Q5E998|CATL2_BOVIN	X			X		
SPARC	P13213|SPRC_BOVIN	X			X		
Alpha-S2-casein	P02663|CASA2_BOVIN	X					
Pantetheinase	Q58CQ9|VNN1_BOVIN	X					
Alpha-2-HS-glycoprotein	P12763|FETUA_BOVIN				X		
Ubiquitin-40S ribosomal protein S27a	P62992|RS27A_BOVIN				X		
Aldose reductase	P16116|ALDR_BOVIN	X	X				
Zinc-alpha-2-glycoprotein	Q3ZCH5|ZA2G_BOVIN	X	X				
Alpha-S1-casein	P02662|CASA1_BOVIN	X	X			X	
Protein DJ-1	Q5E946|PARK7_BOVIN	X	X			X	
Primary amine oxidase, liver isozyme	Q29437|AOCX_BOVIN	X	X		X		
Alpha-1-acid glycoprotein	Q3SZR3|A1AG_BOVIN	X	X		X	X	
Major vault protein	Q3SYU9|MVP_BOVIN	X	X		X	X	
Phosphatidylethanolamine-binding protein 1	P13696|PEBP1_BOVIN	X	X		X	X	
Serpin A3-2	A2I7M9|SPA32_BOVIN	X	X		X	X	
Transthyretin	O46375|TTHY_BOVIN	X	X		X	X	
Vitamin D-binding protein	Q3MHN5|VTDB_BOVIN	X	X		X	X	
Alcohol dehydrogenase [NADP(+)]	Q3ZCJ2|AK1A1_BOVIN				X	X	
Actin, cytoplasmic 1	P60712|ACTB_BOVIN		X		X	X	
Fatty acid-binding protein, heart	P10790|FABPH_BOVIN		X		X	X	
Inositol polyphosphate 1-phosphatase	P21327|INPP_BOVIN		X		X	X	
Nucleoside diphosphate kinase B	Q3T0Q4|NDKB_BOVIN		X		X	X	
Superoxide dismutase [Cu-Zn]	P00442|SODC_BOVIN		X		X	X	
Kininogen-1	P01044|KNG1_BOVIN		X		X		
Triosephosphate isomerase	Q5E956|TPIS_BOVIN		X		X		
Zona pellucida sperm-binding protein 4	Q9BH11|ZP4_BOVIN		X		X		
10 kDa heat shock protein, mitochondrial	P61603|CH10_BOVIN		X			X	
78 kDa glucose-regulated protein	Q0VCX2|GRP78_BOVIN		X			X	
Adenosine deaminase	P56658|ADA_BOVIN		X			X	
Arginase-1	Q2KJ64|ARGI1_BOVIN		X			X	
Beta-lactoglobulin	P02754|LACB_BOVIN		X			X	
Calreticulin	P52193|CALR_BOVIN		X			X	
Glucose-6-phosphate isomerase	Q3ZBD7|G6PI_BOVIN		X			X	
Isocitrate dehydrogenase [NADP] cytoplasmic	Q9XSG3|IDHC_BOVIN		X			X	
Peroxiredoxin-1	Q5E947|PRDX1_BOVIN		X			X	
Peroxiredoxin-2	Q9BGI3|PRDX2_BOVIN		X			X	
Peroxiredoxin-6	O77834|PRDX6_BOVIN		X			X	
Protein disulfide-isomerase	P05307|PDIA1_BOVIN		X			X	
Protein disulfide-isomerase A3	P38657|PDIA3_BOVIN		X			X	
Pterin-4-alpha-carbinolamine dehydratase	Q3ZBD3|PHS_BOVIN		X			X	
Selenium-binding protein 1	Q2KJ32|SBP1_BOVIN		X			X	
Thioredoxin-dependent peroxide reductase, mitochondrial	P35705|PRDX3_BOVIN		X			X	
Ubiquitin carboxyl-terminal hydrolase isozyme L1	P23356|UCHL1_BOVIN		X			X	
Ubiquitin carboxyl-terminal hydrolase isozyme L3	Q2TBG8|UCHL3_BOVIN		X			X	

Description	Accession	26 'excl'	26 'poor'	26 blank	90 'good'	90 'poor'	90 blank
Aspartyl aminopeptidase	Q2HJH1|DNPEP_BOVIN		X				
Aspartate aminotransferase, mitochondrial	P12344|AATM_BOVIN		X				
Beta-casein	P02666|CASB_BOVIN		X				
Bis(5'-adenosyl)-triphosphatase	Q1KZG4|FHIT_BOVIN		X				
Calmodulin	P62157|CALM_BOVIN		X				
Calumenin	Q3T0K1|CALU_BOVIN		X				
Clusterin	P17697|CLUS_BOVIN		X				
Complement factor B	P81187|CFAB_BOVIN		X				
Creatine kinase U-type, mitochondrial	Q9TTK8|KCRU_BOVIN		X				
Desmoglein-1	Q03763|DSG1_BOVIN		X				
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	P11179|ODO2_BOVIN		X				
Glyceraldehyde-3-phosphate dehydrogenase	P10096|G3P_BOVIN		X				
Haptoglobin	Q2TBU0|HPT_BOVIN		X				
Histone H2A type 1	P0C0S9|H2A1_BOVIN		X				
Inositol monophosphatase 1	P20456|IMPA1_BOVIN		X				
Isoaspartyl peptidase/L-asparaginase	Q32LE5|ASGL1_BOVIN		X				
L-lactate dehydrogenase A chain	P19858|LDHA_BOVIN		X				
Malate dehydrogenase, cytoplasmic	Q3T145|MDHC_BOVIN		X				
Nuclear autoantigenic sperm protein	Q2T9P4|NASP_BOVIN		X				
Peptidyl-prolyl cis-trans isomeraseB	P80311|PPIB_BOVIN		X				
Peptidyl-prolyl cis-trans isomerase FKBP1A	P80311|PPIB_BOVIN		X				
Peroxiredoxin-4	Q9BGI2|PRDX4_BOVIN		X				
Phosphoglycerate mutase 1	Q3SZ62|PGAM1_BOVIN		X				
Profilin-1	P02584|PROF1_BOVIN		X				
Rab GDP dissociation inhibitor α	P21856|GDIA_BOVIN		X				
Rho GDP-dissociation inhibitor 1	P19803|GDIR1_BOVIN		X				
Serpin A3-1	Q9TTE1|SPA31_BOVIN		X				
SH3 domain-binding glutamic acid-rich-like protein 3	Q3ZCL8|SH3L3_BOVIN		X				
Stathmin	Q3T0C7|STMN1_BOVIN		X				
Superoxide dismutase [Mn], mitochondrial	P41976|SODM_BOVIN		X				
Thimet oligopeptidase	Q1JPJ8|THOP1_BOVIN		X				
Thymosin beta-4	P62326|TYB4_BOVIN		X				
UPF0556 protein C19orf10 homolog	P62248|CS010_BOVIN		X				
Zona pellucida sperm-binding protein 3	P48830|ZP3_BOVIN		X				
Fatty acid-binding protein, epidermal	P55052|FABP5_BOVIN					X	
Hemoglobin subunit alpha	P01966|HBA_BOVIN					X	
Histone H2B type 1-K	Q2M2T1|H2B1K_BOVIN					X	
L-lactate dehydrogenase B chain	Q5E9B1|LDHB_BOVIN					X	
Macrophage migration inhibitory factor	P80177|MIF_BOVIN					X	
Serum albumin	P02769|ALBU_BOVIN	X	X	X	X	X	X
Insulin	P01317|INS_BOVIN	X	X	X	X	X	X
Serotransferrin	Q29443|TRFE_BOVIN		X	X		X	X

[1] *'Excl = Excellent' embryos: TCN > 64 cells and MDC-ratio = 0% ;* [2] *'Good' embryos: TCN > 64 cells and MDC-ratio = 0.1 – 5 %;* [3] *'Poor' embryos: TCN < 64 cells, MDC-ratio > 90%*

4.4 In *vitro* confirmation of relevance of Cathepsin-L for embryo development

At 45 hpi, cleavage rate was not different between individual culture in ITS or CATH (data not shown), however more embryos reached the 5- to 8-cell stage (fast cleavers) at 45 hpi in CATH compared to ITS (Table 3). Blastocyst rate at 168 hpi was similar for all individual culture systems. However, at 192 hpi, more embryos reached the blastocyst stage when medium was supplemented with Cathepsin-L (CATH), whereas no differences could be observed when the medium was partly refreshed at 80 hpi (CATH-CATH) compared to the control (ITS). No differences could be observed in the hatching rate. Concerning the quality of the obtained blastocysts at 192 hpi, the TCN was similar for all individual culture systems (Table 3), however, the ICM ratio was higher for embryos cultured in medium supplemented with Cathepsin-L (both CATH as CATH-CATH) compared to the control. The apoptotic cell ratio (ACR) was lower in embryos cultured in medium supplemented with Cathepsin-L (CATH), however when culture medium was refreshed with Cathepsin-L (CATH-CATH) no differences could be observed compared to the control (ITS).

5. Discussion

In this study, the secretome of bovine preimplantation embryos using albumin-free culture medium was identified for the first time. By means of a combination of Hoechst/Propidium Iodide staining of the embryos and tandem mass-spectrometry of the embryo-conditioned medium, we were able to detect the proteins actively secreted or released in extracellular vesicles by *in vitro* produced bovine preimplantation embryos of excellent and good quality. Although only the albumin-free culture medium (SOF/PVP/ITS) was used for proteomic analysis, albumin remained the most abundant protein in all analysed samples (Supplementary Tables 1 and 2). Most probably, this albumin was a contamination product of the HEPES-TALP used during the vortex step to remove excessive sperm and cumulus cells after fertilisation. Further reducing the albumin concentration by using albumin-free HEPES-TALP during this step was not attempted, since it strongly affected embryo development in a negative way (unpublished data). We complied with the albumin contamination since we favoured a good equilibrium between embryo development on one side and a reduction of analytical interference and increased ability to detect secreted proteins on the other side. In accordance with the work of Burch and co-workers on the mouse embryo secretome (Burch, et al. 2014), we showed that an albumin-free individual culture approach technically allowed the analysis of secreted proteins.

Table 3. Embryo development and blastocyst quality in individual culture with or without Cathepsin-L supplementation.

		45 hpi (%)	Blastocyst yield (%)			Blastocyst quality[D]		
	n	Fast cleavers[A]	168 hpi[B]	192 hpi[B]	Hatching rate[C]	TCN	ICM-ratio (%)	ACR (%)
ITS	253	**29.7 ± 2.24**	**17.0 ± 2.36**	**22.9 ± 2.64**	**12.1 ± 4.28**	**198.1 ± 7.00**	**29.3 ± 1.13**	**10.7 ± 0.68**
ITS-ITS	251		12.4 ± 2.08	22.7 ± 2.65	10.5 ± 4.06	191.8 ± 9.62	29.4 ± 1.33	10.7 ± 0.69
CATH	252	36.5 ± 2.40 *	17.5 ± 2.39	31.0 ± 2.91 *	5.1 ± 2.49	181.6 ± 9.48	35.9 ± 0.98 *	8.4 ± 0.41 *
CATH-CATH	232		18.5 ± 2.54	28.4 ± 2.96	7.6 ± 3.26	192.4 ± 6.79	35.6 ± 0.91 *	9.0 ± 0.57

[A] *Fast cleavers are expressed as the percentage of embryos that reached the 5-to 8-cell stage relative to the number of cleaved embryos at 45 hpi.*

[B] *Blastocyst yield is expressed as the percentage of blastocysts at 168 and 192 hpi respectively out of the number of presumed zygotes.*

[C] *Hatching rates are expressed as the percentage of hatching or hatched blastocysts at 192 hi compared with the total number of blastocysts.*

[D] *Blastocyst quality was assessed on blastocysts collected at 192 hpi and subjected to differential apoptotic staining: Total cell number (TCN), Inner cell mass ratio (ICM-ratio), as the ratio of the number of cells of the ICM compared to the TCN, and apoptotic cell ratio (ACR) as the ratio of the number of apoptotic cells compared to the TCN.*

Data were compared with the control (individual culture in SOF/BSA/ITS), and differences which were significant ($P < 0.05$) are marked with an asterisk. Data are expressed as mean ± SD.

Autocrine factors can be actively secreted by the blastomeres of preimplantation embryos most probably by means of the constitutive secretory pathway, involving transport of secretory granules to the cell surface and a stimulus-independent release, or by means of extracellular vesicles (for review see Pavani, et al. 2016, Wydooghe, et al. 2017). To confirm that the identified proteins are actively secreted and not passively released by membrane-damaged blastomeres, a fluorescent staining with Hoechst and Propidium Iodide was performed. Surprisingly, only a small percentage of the embryos showed no membrane damaged cells at all, even if the embryos had more than 64 cells at 192 hpi. Almost half of the arrested embryos with less than 64 cells at 192 hpi had a MDC-ratio of more than 50%, indicating that poor quality embryos consist predominantly of membrane-damaged cells that might passively release proteins into the medium. These might be detrimental factors and as such, have a possible negative impact on the surrounding embryos when cultured in group, as argued by proponents of individual culture. Earlier studies demonstrated that especially 'fast cleaving' embryos (5-8 cells at 45 hpi) are prone to negative effects of group culture, since blastocyst development of fast embryos was compromised when less than 30% of the co-cultured embryos reached the blastocyst stage, in comparison to individually cultured 'fast' embryos (OR= 0.3) (Reed, et al. 2011, Wydooghe, et al. 2014b). However, despite this potential downside, it has been demonstrated in several species that the benefits of group culture outweigh the potential negative impacts resulting in better embryo development and quality (for review see Reed, et al. 2011).

For the first time, we were able to identify 4 candidate proteins, actively secreted or released by exosomes, as autocrine factors, which are possibly involved in embryo development. Here, two proteins, SPARC and Cathepsin-L2, caught our special attention since their presence could be confirmed in both the medium of 'excellent' and 'good' embryos. SPARC (secreted protein acidic and rich in cysteine) was initially known as a bone matrix protein, osteonectin or BM-40, involved in bone mineralization by binding Ca^{2+} ions (Termine, et al. 1981). In tumour cells, it has been shown that SPARC inhibits cell-cycle progression (Chetty, et al. 2012) and proliferation by binding to growth factors like vascular endothelial growth factor, basic fibroblast growth factor and platelet derived growth factor (Yan and Sage 1999). Besides, SPARC plays an important role in embryo implantation since suppression of SPARC expression in human trophoblast cells inhibited their invasive capacity and hampered normal placentation (Jiang, et al. 2013). So far, it is not known yet if SPARC plays a role as well during preimplantation embryo development. During preliminary experiments, we were not able to show an effect on bovine embryo development by the addition of SPARC to the culture medium in different concentrations (data not shown).

Cathepsin-L is commonly recognized as a lysosomal protease, involved in intracellular protein catabolism. However, it has been demonstrated that many cancer cells secrete Cathepsin-L into the extracellular milieu, where it plays a role in tumour invasion and metastasis (for review see Gocheva and Joyce 2007). Although tumour invasion is a more destructive process, many similarities do exist between tumour invasion and embryo implantation (for review see Murray and Lessey 1999). It has been hypothesized that Cathepsin-L plays an important role during embryo implantation as well since mouse trophoblast cells express Cathepsin-L (Afonso, et al. 1997). Recently, Cathepsins (A, B, D, L1, L2, Z) have also been identified in the secretome of day 9 and day 10 equine blastocysts, using an LC-MS/MS approach (Swegen, et al. 2017). Besides a role at implantation, it has been demonstrated in sea urchins that the addition of a Cathepsin-L inhibitor to the culture medium during early cleavage cell cycles affected chromosome decondensation and blocked the S-phase of the subsequent cell cycle and as such, affected early preimplantation embryo development (Morin, et al. 2008). The involvement of Cathepsin-L in cell-cycle progression by accelerating S-phase entry has also been shown in mouse fibroblasts (Goulet, et al. 2004) and colorectal carcinoma cells (Tamhane, et al. 2016). Our study is the first to identify Cathepsin-L in the secretome of bovine preimplantation embryos and to evaluate the significance of this identified protein for preimplantation embryo development, by adding human Cathepsin-L (77% homology with CATL2_BOVIN and 100% homology for active sites – UNIPROT) to the culture medium of individually cultured bovine embryos. Based on our results, we hypothesize that Cathepsin-L is also involved in cell-cycle progression during first cleavages of bovine embryonic development, since more embryos reached the third cleavage division (5-to 8-cell stage) already at 45 hpi in the presence of Cathepsin-L compared to other test groups. Furthermore, blastocyst rate of the Cathepsin-L-supplemented individual culture at 192 hpi was higher compared to the blastocyst rate in the absence of Cathepsin-L. Nevertheless, after embryonic genome activation, Cathepsin-L could not hasten cell-cycle progression in bovine embryos, blastocyst development in the presence of Cathepsin-L was similar at 168 hpi compared to individual culture without Cathepsin-L supplementation, and at 192 hpi, hatching rate was similar. Refreshing the culture medium with Cathepsin-L supplementation after embryonic genome activation could not improve blastocyst development nor hatching rate. Looking at the quality of the obtained blastocysts, total cell number in individual culture with or without Cathepsin-L was similar. However, the addition of Cathepsin-L to the culture medium resulted in a higher ICM-ratio when compared to standard culture medium. Furthermore, the ACR in the individual culture in the presence of Cathepsin-L was lower when compared to the individual culture without Cathepsin-L supplementation. We did not confirm if Cathepsin-L was incorporated in the embryos so the mode of action (intracellularly or extracellularly) of Cathepsin-L in culture medium remains to be elucidated.

Two other proteins could only be identified in the pooled medium of 26 'excellent' embryos, whereas their presence could not be confirmed in the pooled medium of 90 'good' embryos. Since these 2 pools of medium could be considered as 2 replicates, we did not further explore the relevance of these proteins as their presence was not confirmed in both samples. Shortly, Alpha-S2-casein is best known as a component of milk which has been shown to have an anti-oxidant activity (Aparna, et al. 2010) however to the best of our knowledge no possible role on embryo development has been elucidated yet. Pantetheinase is an ubiquitous enzyme located on the cell membrane which hydrolyses D-pantetheine an intermediate metabolite of coenzyme A, into pantothenic acid (vitamin B5) and cysteamine, a potent antioxidant (Nitto, et al. 2008). Recently, pantetheinase has been identified in the bovine uterine fluid collected during the preimplantation phase (Mullen, et al. 2012), and in the exosomes released by bovine endometrial stromal cells (Koh, et al. 2016) suggesting a putative role in supporting early embryo development and implantation.

In conclusion, we have identified possible embryotrophic proteins by means of tandem mass spectrometry using individual culture of bovine embryos in albumin-free culture medium. We confirmed a critical role for Cathepsin-L on preimplantation embryo development as Cathepsin-L supplementation to the culture medium of individually cultured bovine embryos was (1) progressing the first cell divisions, and (2) both blastocyst development as well as (3) blastocyst quality improved compared to individual culture in the absence of Cathepsin-L. Further research should focus on the effect of the other identified proteins: not only the possible positive effect of proteins identified in the medium of excellent and good embryos, but also the effect (positive or negative) of the proteins identified in the medium of bad embryos. Furthermore, the optimal way of supplementing these proteins to the culture medium should be evaluated. For example, it might be interesting to add the proteins encapsulated in exosome-like structures to prevent degradation in the culture medium and as such, increasing their accessibility to the embryos. This paper provides a rich resource for further studies on inter-embryo communications. The sum of the knowledge acquired by means of transcriptomics, proteomics, lipidomics and metabolomics will be a helpful tool to further optimize *in vitro* embryo culture conditions, both for human and bovine embryos.

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Supplementary Table 1. Proteins identified in a pool of SOF/PVP/ITS medium conditioned by 90 embryos of 'good' quality (TCN > 64 cells; MDC-ratio = 0.1 - 5%) and 90 embryos of 'poor' quality (TCN < 64 cells; MDC-ratio > 90%).

	Accession	Score[a]	# Peptides[b] ($p < 0.01$)	# Sequences ($p < 0.01$)	Description
'Good' quality embryos: TCN > 64 cells; MDC-ratio = 0.1 - 5 %					
1	P02769|ALBU_BOVIN	8792	242	58	Serum albumin
2	O46375|TTHY_BOVIN	967	21	11	Transthyretin
3	Q3SZR3|A1AG_BOVIN	458	17	8	Alpha-1-acid glycoprotein
4	Q3SYU9|MVP_BOVIN	453	13	10	Major vault protein
5	P13696|PEBP1_BOVIN	436	10	7	Phosphatidylethanolamine-Binding prot. 1
6	A2I7M9|SPA32_BOVIN	298	8	6	Serpin A3-2
7	Q3MHN5|VTDB_BOVIN	298	7	7	Vitamin D-binding protein
8	P01317|INS_BOVIN	231	12	2	Insulin
9	Q5E998|CATL2_BOVIN	219	5	4	Cathepsin-L2
10	Q29437|AOCX_BOVIN	194	6	5	Primary amine oxidase, liver isozyme
11	P10790|FABPH_BOVIN	129	2	2	Fatty acid-binding protein, heart
12	Q3ZCJ2|AK1A1_BOVIN	120	3	3	Alcohol dehydrogenase [NADP(+)]
13	Q3T0Q4|NDKB_BOVIN	117	3	2	Nucleoside diphosphate kinase B
14	P00442|SODC_BOVIN	117	2	2	Superoxide dismutase [Cu-Zn]
15	P01044|KNG1_BOVIN	92	3	3	Kininogen-1
16	Q5E956|TPIS_BOVIN	86	2	2	Triosephosphate isomerase
17	Q9BH11|ZP4_BOVIN	82	2	2	Zona pellucida sperm-binding protein 4
18	P12763|FETUA_BOVIN	72	2	2	Alpha-2-HS-glycoprotein
19	P13213|SPRC_BOVIN	69	4	4	SPARC
20	P21327|INPP_BOVIN	64	2	2	Inositol polyphosphate 1-phosphatase
21	P62992|RS27A_BOVIN	55	2	2	Ubiquitin-40S ribosomal protein S27a
22	P60712|ACTB_BOVIN	33	2	2	Actin, cytoplasmic 1
'Poor' quality embryos: TCN < 64 cells; MDC-ratio > 90 %					
1	P02769|ALBU_BOVIN	5107	146	43	Serum albumin
2	Q3SYU9|MVP_BOVIN	1481	45	34	Major vault protein
3	O46375|TTHY_BOVIN	668	13	8	Transthyretin
4	P13696|PEBP1_BOVIN	508	9	7	Phosphatidylethanolamine-binding prot. 1
5	Q9BGI3|PRDX2_BOVIN	399	10	7	Peroxiredoxin-2
6	P60712|ACTB_BOVIN	313	9	7	Actin, cytoplasmic 1
7	P21327|INPP_BOVIN	258	6	5	Inositol polyphosphate 1-phosphatase
8	P01317|INS_BOVIN	257	12	2	Insulin
9	Q3SZR3|A1AG_BOVIN	249	9	9	Alpha-1-acid glycoprotein
10	Q3MHN5|VTDB_BOVIN	238	6	5	Vitamin D-binding protein
11	Q0VCX2|GRP78_BOVIN	232	6	6	78 kDa glucose-regulated protein
12	Q5E947|PRDX1_BOVIN	224	9	6	Peroxiredoxin-1
13	A2I7M9|SPA32_BOVIN	218	6	4	Serpin A3-2
14	P10790|FABPH_BOVIN	188	3	3	Fatty acid-binding protein, heart
15	P56658|ADA_BOVIN	157	5	4	Adenosine deaminase
16	P02754|LACB_BOVIN	149	2	2	Beta-lactoglobulin
17	P02662|CASA1_BOVIN	143	3	3	Alpha-S1-casein
18	Q29443|TRFE_BOVIN	142	6	2	Serotransferrin
19	Q3ZBD7|G6PI_BOVIN	140	3	2	Glucose-6-phosphate isomerase
20	P23356|UCHL1_BOVIN	138	3	3	Ubiquitin carboxyl-terminal hydrolase isozyme L1
21	Q3T0Q4|NDKB_BOVIN	124	5	4	Nucleoside diphosphate kinase B
22	Q3ZCJ2|AK1A1_BOVIN	122	4	4	Alcohol dehydrogenase [NADP(+)]
23	P38657|PDIA3_BOVIN	117	5	5	Protein disulfide-isomerase A3
24	Q9XSG3|IDHC_BOVIN	114	2	2	Isocitrate dehydrogenase [NADP] cytoplasmic

25	P61603|CH10_BOVIN	108	2	2	10 kDa heat shock protein, mitochondrial
26	Q5E9B1|LDHB_BOVIN	107	3	3	L-lactate dehydrogenase B chain
27	Q5E946|PARK7_BOVIN	105	2	2	Protein DJ-1
28	Q2KJ32|SBP1_BOVIN	87	2	2	Selenium-binding protein 1
29	P35705|PRDX3_BOVIN	84	2	2	Thioredoxin-dependent peroxide reductase, mitochondrial
30	Q2KJ64|ARGI1_BOVIN	84	2	2	Arginase-1
31	Q2M2T1|H2B1K_BOVIN	76	2	2	Histone H2B type 1-K
32	O77834|PRDX6_BOVIN	74	2	2	Peroxiredoxin-6
33	P00442|SODC_BOVIN	66	2	2	Superoxide dismutase [Cu-Zn]
34	P80177|MIF_BOVIN	64	2	2	Macrophage migration inhibitory factor
35	Q3ZBD3|PHS_BOVIN	63	2	2	Pterin-4-alpha-carbinolamine dehydratase
36	P55052|FABP5_BOVIN	57	2	2	Fatty acid-binding protein, epidermal
37	P52193|CALR_BOVIN	56	3	3	Calreticulin
38	Q2TBG8|UCHL3_BOVIN	55	2	2	Ubiquitin carboxyl-terminal hydrolase isozyme L3
39	P01966|HBA_BOVIN	50	2	2	Hemoglobin subunit alpha
40	P05307|PDIA1_BOVIN	47	2	2	Protein disulfide-isomerase

[a] *The score of a peptide is a measure for the quality of the spectrum obtained after MSMS.*

[b] *The number of identified peptides is mentioned as a rough estimate of the abundance of this protein in the sample.*

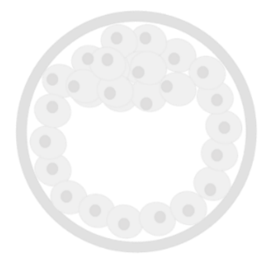
Supplementary Table 2. Proteins identified in a pool of SOF/PVP/ITS medium conditioned by 26 embryos of 'excellent' quality (TCN > 64 cells; MDC-ratio = 0%) and 26 embryos of 'poor' quality (TCN < 64 cells; MDC-ratio > 90%).

	Accession	Score[a]	# Peptides[b] (p < 0.01)	# Sequences (p < 0.01)	Description
'Excellent' quality embryos: TCN > 64 cells; MDC-ratio = 0 %					
1	P02769|ALBU_BOVIN	31115	1117	47	Serum albumin
2	Q3SZR3|A1AG_BOVIN	669	25	7	Alpha-1-acid glycoprotein
3	P01317|INS_BOVIN	667	58	2	Insulin
4	Q3MHN5|VTDB_BOVIN	579	18	7	Vitamin D-binding protein
5	P02662|CASA1_BOVIN	571	17	4	Alpha-S1-casein
6	O46375|TTHY_BOVIN	541	13	4	Transthyretin
7	A2I7M9|SPA32_BOVIN	333	10	5	Serpin A3-2
8	Q3ZCH5|ZA2G_BOVIN	248	7	5	Zinc-alpha-2-glycoprotein
9	Q29437|AOCX_BOVIN	147	3	3	Primary amine oxidase, liver isozyme
10	P13696|PEBP1_BOVIN	137	7	3	Phosphatidylethanolamine-binding protein 1
11	Q58CQ9|VNN1_BOVIN	109	3	3	Pantetheinase
12	Q3SYU9|MVP_BOVIN	83	3	3	Major vault protein
13	P16116|ALDR_BOVIN	64	2	2	Aldose reductase
14	P13213|SPRC_BOVIN	57	2	2	SPARC
15	P02663|CASA2_BOVIN	54	4	2	Alpha-S2-casein
16	Q5E998|CATL2_BOVIN	49	4	3	Cathepsin-L2
17	Q5E946|PARK7_BOVIN	43	2	2	Protein DJ-1
'Poor' quality embryos: TCN < 64 cells; MDC-ratio > 90 %					
1	P02769|ALBU_BOVIN	20156	627	63	Serum albumin
2	Q3SYU9|MVP_BOVIN	1973	54	29	Major vault protein
3	O46375|TTHY_BOVIN	1520	31	8	Transthyretin
4	P13696|PEBP1_BOVIN	1358	35	11	Phosphatidylethanolamine-binding protein 1
5	P01317|INS_BOVIN	990	75	2	Insulin
6	Q3SZR3|A1AG_BOVIN	971	31	9	Alpha-1-acid glycoprotein
7	Q3MHN5|VTDB_BOVIN	738	21	10	Vitamin D-binding protein
8	Q3ZBD7|G6PI_BOVIN	643	15	11	Glucose-6-phosphate isomerase
9	P21327|INPP_BOVIN	599	19	10	Inositol polyphosphate 1-phosphatase
10	P00442|SODC_BOVIN	538	9	4	Superoxide dismutase [Cu-Zn]
11	Q9BGI3|PRDX2_BOVIN	501	11	8	Peroxiredoxin-2
12	Q3T0Q4|NDKB_BOVIN	488	14	6	Nucleoside diphosphate kinase B
13	O77834|PRDX6_BOVIN	448	12	10	Peroxiredoxin-6
14	P52193|CALR_BOVIN	441	10	7	Calreticulin
15	P38657|PDIA3_BOVIN	394	13	12	Protein disulfide-isomerase A3
16	P19858|LDHA_BOVIN	367	11	8	L-lactate dehydrogenase A chain
17	P10790|FABPH_BOVIN	332	9	6	Fatty acid-binding protein, heart
18	Q0VCX2|GRP78_BOVIN	322	9	7	78 kDa glucose-regulated protein
19	P23356|UCHL1_BOVIN	287	5	4	Ubiquitin carboxyl-terminal hydrolase isozyme L1
20	P60712|ACTB_BOVIN	283	10	6	Actin, cytoplasmic 1
21	A2I7M9|SPA32_BOVIN	282	10	9	Serpin A3-2
22	Q9TTE1|SPA31_BOVIN	282	10	9	Serpin A3-1
23	Q3T0K1|CALU_BOVIN	281	6	4	Calumenin
24	P56658|ADA_BOVIN	275	8	8	Adenosine deaminase
25	Q9TTK8|KCRU_BOVIN	273	6	5	Creatine kinase U-type, mitochondrial
26	Q9XSG3|IDHC_BOVIN	266	10	10	Isocitrate dehydrogenase [NADP] cytoplasmic
27	P16116|ALDR_BOVIN	266	7	5	Aldose reductase
28	Q2HJH1|DNPEP_BOVIN	241	5	5	Aspartyl aminopeptidase
29	P0C0S9|H2A1_BOVIN	236	4	2	Histone H2A type 1
30	P02754|LACB_BOVIN	220	3	2	Beta-lactoglobulin

31	P05307|PDIA1_BOVIN	218	6	6	Protein disulfide-isomerase
32	P62157|CALM_BOVIN	217	4	2	Calmodulin
33	P62326|TYB4_BOVIN	183	6	2	Thymosin beta-4
34	Q5E946|PARK7_BOVIN	177	5	5	Protein DJ-1
35	Q5E956|TPIS_BOVIN	176	4	3	Triosephosphate isomerase
36	Q2T9P4|NASP_BOVIN	159	4	3	Nuclear autoantigenic sperm protein
37	Q2KJ64|ARGI1_BOVIN	155	6	5	Arginase-1
38	Q2KJ32|SBP1_BOVIN	154	4	4	Selenium-binding protein 1
39	P61603|CH10_BOVIN	151	3	3	10 kDa heat shock protein, mitochondrial
40	P02662|CASA1_BOVIN	151	6	3	Alpha-S1-casein
41	Q5E947|PRDX1_BOVIN	143	7	6	Peroxiredoxin-1
42	Q9BGI2|PRDX4_BOVIN	98	4	3	Peroxiredoxin-4
43	P02666|CASB_BOVIN	138	4	2	Beta-casein
44	Q3ZBD3|PHS_BOVIN	128	5	4	Pterin-4-alpha-carbinolamine dehydratase
45	P10096|G3P_BOVIN	126	3	2	Glyceraldehyde-3-phosphate dehydrogenase
46	P41976|SODM_BOVIN	125	4	2	Superoxide dismutase [Mn], mitochondrial
47	Q3ZCH5|ZA2G_BOVIN	125	5	5	Zinc-alpha-2-glycoprotein
48	Q2TBG8|UCHL3_BOVIN	120	6	3	Ubiquitin carboxyl-terminal hydrolase isozyme L3
49	Q3T145|MDHC_BOVIN	120	6	6	Malate dehydrogenase, cytoplasmic
50	P80311|PPIB_BOVIN	112	3	2	Peptidyl-prolyl cis-trans isomerase FKBP1A
51	Q9BH11|ZP4_BOVIN	109	2	2	Zona pellucida sperm-binding protein 4
52	Q29443|TRFE_BOVIN	108	6	4	Serotransferrin
53	P02584|PROF1_BOVIN	104	3	3	Profilin-1
54	Q32LE5|ASGL1_BOVIN	102	3	3	Isoaspartyl peptidase/L-asparaginase
55	Q1KZG4|FHIT_BOVIN	100	3	3	Bis(5'-adenosyl)-triphosphatase
56	P11179|ODO2_BOVIN	98	3	2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial
57	Q3SZ62|PGAM1_BOVIN	97	5	4	Phosphoglycerate mutase 1
58	Q1JPJ8|THOP1_BOVIN	96	3	3	Thimet oligopeptidase
59	P17697|CLUS_BOVIN	95	2	2	Clusterin
60	P12344|AATM_BOVIN	89	4	3	Aspartate aminotransferase, mitochondrial
61	Q29437|AOCX_BOVIN	87	3	3	Primary amine oxidase, liver isozyme
62	P81187|CFAB_BOVIN	81	2	2	Complement factor B
63	Q03763|DSG1_BOVIN	77	3	3	Desmoglein-1
64	P20456|IMPA1_BOVIN	74	3	3	Inositol monophosphatase 1
65	P35705|PRDX3_BOVIN	71	3	2	Thioredoxin-dependent peroxide reductase, mitochondrial
66	P19803|GDIR1_BOVIN	71	2	2	Rho GDP-dissociation inhibitor 1
67	P80311|PPIB_BOVIN	71	4	3	Peptidyl-prolyl cis-trans isomerase B
68	P48830|ZP3_BOVIN	67	2	2	Zona pellucida sperm-binding protein 3
69	P62248|CS010_BOVIN	67	2	2	UPF0556 protein C19orf10 homolog
70	P12344|AATM_BOVIN	67	4	3	Aspartate aminotransferase, cytoplasmic
71	P21856|GDIA_BOVIN	64	3	3	Rab GDP dissociation inhibitor alpha
72	Q3T0C7|STMN1_BOVIN	61	2	1	Stathmin
73	Q2TBU0|HPT_BOVIN	50	3	3	Haptoglobin
74	P01044|KNG1_BOVIN	48	2	2	Kininogen-1
75	Q3ZCL8|SH3L3_BOVIN	47	2	2	SH3 domain-binding glutamic acid-rich-like protein 3

[a] *The score of a peptide is a measure for the quality of the spectrum obtained after MSMS.*

[b] *The number of identified peptides is mentioned as a rough estimate of the abundance of this protein in the sample*



CHAPTER 7

GENERAL DISCUSSION

1. *In vitro* embryo production: is teamwork making the dream work?

An embryo is able to promote its own development, whether or not mom is around. However in terms of viability, embryos produced *in vitro* are consistently lagging behind their *in vivo* produced counterparts, as evidenced by lower pregnancy rates (Fleming, et al. 2004). Obtaining a pregnancy and giving birth to healthy offspring is the ultimate goal of an *in vitro* fertility treatment, therefore, many efforts have been made to improve the *in vitro* culture conditions ever since the start of IVF. The focus of this thesis is to investigate and identify how inter-embryonic communication of bovine embryos occurs when cultured in group. Based on this knowledge, we want to optimize the composition of the medium that is used to culture the embryos from zygote to the blastocyst stage. In this thesis a novel approach has been used: embryo conditioned medium was subjected to tandem mass spectrometry in order to perform a screening of all the proteins that are secreted by bovine preimplantation embryos. These proteins are possible candidates to be autocrine factors, which are factors produced and released by the embryo itself and act upon the embryo or the neighbouring embryos. The presence of these autocrine factors is believed to be the reason for superior development in group culture compared to individual culture as described for bovine (Donnay, et al. 1997, Goovaerts, et al. 2009, O'Doherty, et al. 1997, Wydooghe, et al. 2014), mouse (Canseco, et al. 1992, Lane and Gardner 1992, Paria and Dey 1990, Salahuddin, et al. 1995), pig (Stokes, et al. 2005) and cat (Spindler and Wildt 2002) embryos.

As pointed out in **Chapter 1**, bovine embryos represent an attractive model for human embryos, which are not often used for research purposes because of ethical restrictions. However at first, the culture system used standardly for those two species is very different. In bovine embryos, group culture is the method of choice since single embryo culture is dramatically reducing development from a typical 30-40 % of blastocysts in group culture (Lonergan 2007), towards less than 5 % of blastocysts in single culture (Donnay, et al. 1997, Goovaerts, et al. 2009, O'Doherty, et al. 1997). This is in contrast to human settings, where individual culture of embryos is the preferred method mainly because human embryo quality assessment requires individual follow-up during the entire culture period. Fortunately, individual culture of human embryos is leading to better results compared to bovine individual culture, since on average 30% of human embryos will reach the blastocyst stage at day 5 (Papanikolaou, et al. 2006). When the general idea for this thesis was drafted in 2009, in most cases foetal bovine serum (FBS) was supplemented to bovine culture medium in order to provide growth factors and energy sources. However, in this thesis FBS had to be omitted from the culture medium since it is an undefined and rich source of proteins that would make the proteomic profile of the embryo conditioned culture medium very complex. Unexpectedly, we showed in **chapter 4** that

using serum-free culture medium based on bovine serum albumin (BSA) and insulin, transferrin and selenium (ITS), led to high blastocyst development of individually cultured bovine embryos: blastocyst rate was 35.2% at 192 hpi in individual culture, which is similar to 36.5% in group culture. This evolution increased the value of the bovine embryo culture system as a model for human IVF, since comparable results could now be obtained in individual culture of embryos. Furthermore, we could show in **chapter 4** that, although there was no difference in the development of embryos cultured individually or in groups, the quality of the blastocysts obtained in group culture was better compared to individual culture. During the course of this thesis, we experienced that the individual culture system was much more sensitive to small changes in the culture system as e.g. temperature fluctuations in the lab. Therefore, we believe that human embryos may also take advantage of the autocrine secretions present in group culture systems, and this will hopefully lead to increased embryo quality and pregnancy outcome.

As discussed in **chapter 5**, new group culture devices have been designed for human embryos that allow individual follow-up of the embryos throughout the whole culture period. We showed that Primo Vision® dishes provide an excellent system that combines the benefits of group and individual culture, leading to a similar rate of embryo development compared to classical group culture and gives better embryo development and quality than individual culture or culture in Corral® dishes. The Primo Vision® dishes are currently used in human IVF labs in combination with time-lapse cinematography, however up till now, is not yet the standard culture system in the routine practice. Proponents of individual culture of human embryos argue that, regardless of the production of autocrine factors, embryos in groups may be exposed to negative effects of dying or delayed embryos cultured in the same drop (Reed, et al. 2011). However, these arguments can be contradicted since by using the Primo Vision® dish, we showed that non-cleaved or arrested embryos do not hamper the ability of co-cultured bovine embryos to reach the blastocyst stage in group culture. More novel insights on the effect of group culture are the fact that a higher number of ‘slow’ embryos developed to the blastocyst stage compared to their individually cultured counterparts, while no differences were observed for ‘fast’ embryos. Based on these data new concerns could be raised about group culture systems rescuing embryos of inferior quality, however more research is needed to compare the quality of the obtained blastocysts in terms of pregnancy rate, live birth rate, and health of the offspring.

Therefore, a clinical trial was designed to compare individual human embryo culture and group culture using the Primo Vision® dish, based on the results obtained in **chapter 5**. It was our hypothesis that by using a group culture system, as the Primo Vision® dish, human embryos cultured in group can benefit from autocrine factors of different origin (as discussed in **chapter 1**). The aim of this study was

to increase the utilisation rate, calculated as the number of blastocysts suitable for transfer or cryopreservation out of the number of zygotes (2PN), from 40% in individual culture to 60% by culturing the embryos in group in Primo Vision® dishes. Therefore, a multicentre (UZ Ghent + UZ Antwerp) prospective study on sibling oocytes was conducted, in which 158 patients had to be included of which half of the zygotes would be cultured individually (standard culture system) and half of the zygotes would be cultured in group in a Primo Vision® dish, both until 116 ± 1 hpi. Patients with at least 10 zygotes (2PN) could be included in the study after informed consent, and randomly half of the zygotes were allocated to individual culture and the other half to group culture in Primo Vision® dishes. In both culture systems, embryos were cultured until 68 ± 1 hpi in a drop of Cook Cleavage medium (20 µl for individual culture, 30µl in a Primo Vision® dish; no medium refreshment), and afterwards transferred to a drop of Cook Blastocyst medium until 116 ± 1 hpi (20 µl for individual culture, 30µl in a Primo Vision® dish; no medium refreshment). Daily evaluation of the embryo development was performed. Unfortunately, the first interim analysis (27 patients included), showed that the utilisation rate was lower, although not significantly (p = 0.324), when embryos were cultured in small groups compared to individually cultured human embryos (Table 1). Concerns were uttered about the lower number of embryos that could be transferred at 68 ± 1 hpi to Cook Blastocyst medium in Primo Vision® plates compared to individual culture. However, looking at the number of blastocysts with a score 3 or more (according to Gardner and Schoolcraft 1999), the difference between individual and group culture was very small and not significant in the end.

Table 1. Interim analysis of the multicentre clinical randomized trial.

		INDIVIDUAL		**PRIMO VISION®**	
	Time point	No.	% (x / 2 PN)	No.	% (x / 2 PN)
2 PN	20 hpi	161	-	167	-
TR	68 hpi	129	**80.1** [a]	117	**70.1** [b]
Blast.	116 hpi	89	55.3	84	50.3
Blast. (≥ 3)	116 hpi	56	34.8	53	31.7
UT	116 hpi	65	40.6	59	35.3

Differences at P < 0.05 were considered to be significant and are marked with a different superscript. Abbreviations: TR, embryos that could be transferred on day 3; Blast, blastocysts; Blast (≥ 3), blastocysts with a score 3 or more (Gardner and Schoolcraft, 1999); PN, pronuclei; UT, embryos that can be used for uterine transfer or cryopreservation.

Based on the interim analysis, we decided to stop the clinical trial. In contrast to bovine embryos, human embryo development seemed not to improve in group culture based on our results. In literature, contradictory results have been published on possible advantages for human embryos in group culture (Almagor, et al. 1996, Ebner, et al. 2010, Moessner and Dodson 1995, Restelli, et al. 2014, Rijnders and Jansen 1999, Spyropoulou, et al. 1999, Tao, et al. 2013). Unfortunately, these studies are difficult to compare because of differences in embryo culture conditions, different outcome parameters reported or no comparison to individual culture is performed. Ebner et al. (2010), which is the most recent study comparing group culture and individual culture, showed that human embryos do benefit from group culture in terms of compaction and blastulation rates and blastocyst quality as compared to individual culture. As a trend, more life births were achieved with blastocysts derived from group culture. When using Primo Vision® dishes, we could not show a benefit of group culture compared to individual culture. Although microwells have been shown to improve embryo development in bovine, this could be different in human embryos. Culture in microwells alters gene expression of bovine embryos compared to standard group culture, however it remains unclear whether these changes correlate with increased or decreased embryo quality (Hoelker, et al. 2009). The effect of the differentially expressed genes is controversial and the net outcome might be different in bovine versus human embryos. Furthermore, it has been concluded that a micro-environmental embryo density of 1/0.269 µl and a macro-environmental embryo density of 1/30 µl were most successful for culturing bovine embryos in WOW dishes (Hoelker, et al. 2010): whether this is similar for human embryos is not known. The embryo density used in our study depends on the number of 2 PNs of the patient (5 - 9 embryos in 30 µl medium: 1/6 – 1/3.3 µl) and was different from the density in the study of Ebner (3 embryos in 30µl medium: 1/10 µl). For bovine and mouse embryos an embryo density of one embryo per 2 µl medium has been described as the optimum density to maximize the effects of autocrine factors (Kato and Tsunoda 1994, Palasz and Thundathil 1998). The optimal embryo density for human embryos remains to be elucidated with a good equilibrium between the positive effects of the autocrine factors and the accumulation of possible waste products. This might be different for each type of medium that can be used in human IVF systems, since different commercially prepared media can be used with slightly different compositions. In bovine embryos, we could confirm that the combination of 2-step COOK medium and Primo Vision® dishes was not supporting embryo development to the same extent as SOF/BSA/ITS and Primo Vision® dishes (Blastocyst rate at 192 hpi: 12.1% vs 30.3% respectively; n= 198). Pregnancy rate and live birth rate were secondary parameters of our study, however these data were to sparse at the time of the in the interim analysis, likewise, we cannot draw any conclusions on the viability of the obtained blastocyst.

Using a group culture system might be an important consideration over the provision of better medium ingredients, in terms of identified secreted proteins. The group culture system mimics more the dynamic and complex nature of *in vivo* conditions compared to static (bovine) or 2-step (human) culture media. However, based on the interim analysis, we decided to stop the clinical trial and focus on the identification of autocrine factors by means of proteomics. Likewise, by addition of these embryotrophic factors, the culture medium of bovine and human embryos can be optimised.

2. Bovine embryo secretomics: looking for a needle in a haystack

The first attempts to identify autocrine factors released by preimplantation embryos were by means of transcriptomics, by reverse transcription polymerase chain reaction (RT-PCR) (Watson, et al. 1992). However, protein abundance cannot by reliably predicted from transcript abundance, and the biochemical function of a protein frequently requires post-translational modifications. Therefore, several proteomic approaches have been undertaken to unravel inter-embryo communication. The oldest technique for protein identification is by using specific antibodies, for example by enzyme linked immunosorbent assay (ELISA), soluble human leucocyte antigen-G (sHLA-G) could be identified in culture medium. Another technique is 2D-polyacrylamide gel electrophoresis (2D-PAGE), separating the proteins of complex samples by two orthogonal physicochemical parameters (i.e. isoelectric point and molecular mass of a protein). Quantification of 2D-PAGE spot intensity is performed either by scanner-based absorbance spectroscopy after staining the gel with Coomassie blue or by using a fluorescent staining. However, this approach requires a large amount of starting material, is complex and labour intensive (For review see Arnold and Frohlich 2011).

The latest developed technique, only available since the early nineties, is mass spectrometry based protein identification. By using mass spectrometry it is possible to perform a complete screening of all proteins secreted by preimplantation embryos at once. Katz-Jaffe and co-workers used the surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF MS) to generate protein profiles of mouse and human embryos throughout development (Katz-Jaffe, et al. 2005, Katz-Jaffe, et al. 2006). They demonstrated that embryos secrete characteristic protein profiles at each 24-hour interval from fertilisation till blastocyst stage. Furthermore, the expression of an 8.5-kDa protein biomarker, for which the best candidate was ubiquitin, increased toward the blastocyst stage and it was the most abundant protein in the day 5 secretome. Interestingly, it was observed that embryos of normal morphology released more of this protein than those with signs of degeneration, suggesting this might be a non-invasive biomarker for the developmental potential of human embryos generated *in vitro* (Katz-Jaffe, et al. 2006). However, an important limitation of the SELDI-TOF technology is that the protein peaks cannot be identified conveniently. An alternative proteomic

strategy, which was also attempted in this thesis, is based on the combination of high performance liquid chromatography (HPLC) system coupled with nano-electrospray ionization tandem mass spectrometry (LC-MS/MS) analysis of tryptic digests of the culture media. Beardsley and co-workers (2010) compared the suitability of both techniques (e.g. SELDI-TOF and LC-MS/MS) to analyse the secretome of mouse preimplantation embryos. By means of SELDI-TOF, only one protein peak could be observed in the embryo conditioned medium, at ~ 8570 m/z, which is similar to the peak of 8500 Da earlier described (Katz-Jaffe, et al. 2006), however no additional peaks could be identified. Whereas with LC-MS/MS a total of 20 putative proteins could be identified, indicating that LC-MS/MS is a more sensitive tool to study the secretome of preimplantation embryos (Beardsley, et al. 2010).

In this thesis, LC-MS/MS was used to identify the secretome of bovine preimplantation embryos. The main challenges to tackle in this part of the thesis, were the technical limitations in LC/MSMS in the detection of the low-abundant proteins of interest against the dominance of albumin, which is added at a concentration of 4 mg/ml in the culture medium that is used in this thesis (SOF/BSA/ITS). In order to get rid of the albumin added to the culture medium, at first, a SDS-PAGE gel electrophoresis was performed prior to the analysis on mass spectrometry. From the spent culture medium and the blank medium, 3 different samples were prepared in order to have a protein content of 100 µg, 50 µg and 20 µg, and in addition a sample of 1 µg pure BSA. After gel electrophoresis the bands above and under the band of albumin were cut out (Figure 1), followed by in gel trypsin digest and protein identification by mass spectrometric analysis. Besides keratins, most probably contamination products from handling of the gel, only 3 proteins were confidently identified (e.g. a peptide p-value < 0.01 in Mascot Deamon and minimal 2 sequences per protein): serum albumin (score 3966, 109 peptides), alpha-1-acid glycoprotein (score 368, 10 peptides) and alpha-S1-casein (score 40, 3 peptides). Despite the albumin depletion step with a SDS PAGE and the exclusion list, albumin remains the most abundant protein in the spent culture medium and proteins of interest remained anonymous.

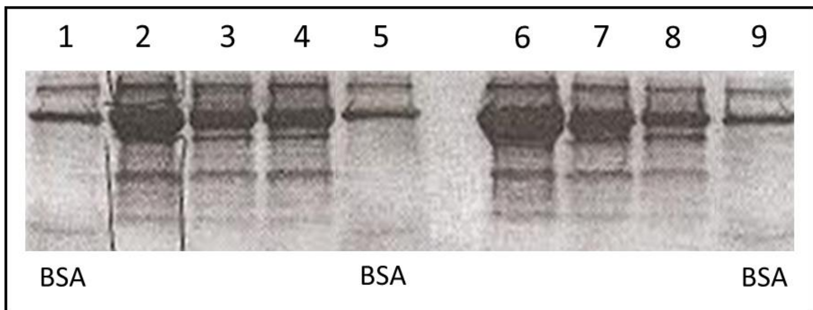


Figure 1. *SDS-PAGE gel electrophoresis of spent embryo culture medium (protein content 100 µg, 50 µg and 20 µg) and blanc culture medium (protein content 100 µg, 50 µg and 20 µg). In addition a sample of 1 µg pure BSA was added in line 1, 5 and 9.*

In murine embryos, it was demonstrated that using albumin-free embryo culture medium led to a higher number of protein identifications compared to culture medium with albumin supplementation (Burch, et al. 2014). Therefore, we tested the feasibility of defined culture medium, in which BSA in SOFaa was replaced by 0.1 mg/ml Polyvinylpyrrolidone (SOF/ITS/PVP), in order to identify proteins secreted by bovine embryos. Since we had no idea whether this culture medium would support embryonic development sufficiently, we decided to split the culture period around 80 hpi or the moment of embryonic genome activation (Figure 2). Likewise we could collect 4 different samples for protein analysis: blank medium (Figure 2, line A), PVP till 192 hpi (Figure 2, line B), PVP till 80 hpi (Figure 2, line C) and PVP 80 - 192 hpi (Figure 2, line D).

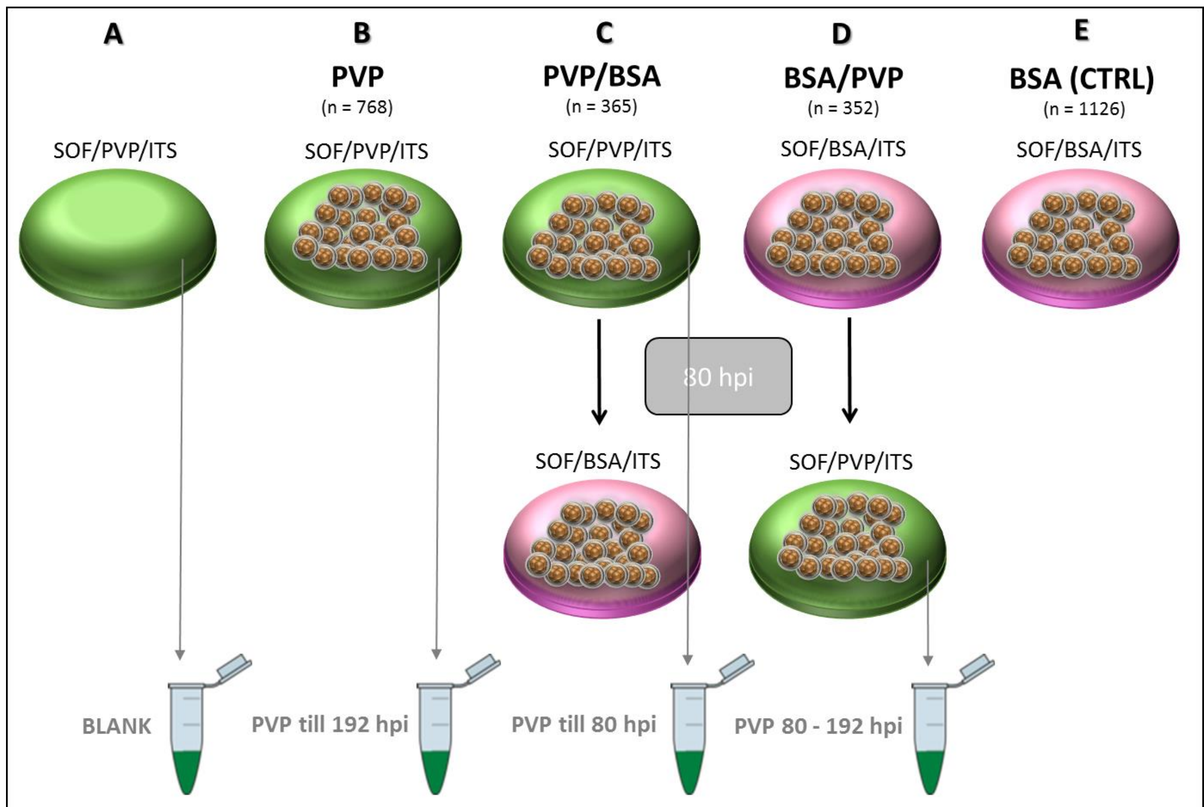


Figure 2. *Experimental setup of group culture with defined medium. Green cultures: PVP/ITS; pink cultures: BSA/ITS. (A) medium without embryos was placed in the incubator, until 192 hpi when medium was collected (BLANK); (B) PVP: embryos were cultured the whole culture period in PVP/ITS; at 192 hpi embryos were removed and medium was collected (PVP till 192 hpi); (C) PVP/BSA: embryos were cultured in PVP/ITS until 80 hpi, medium was collected (PVP till 80 hpi) and embryos were transferred to BSA/ITS; (D) BSA/PVP: embryos were cultured in BSA/ITS until 80hpi, and subsequently embryos were transferred to PVP/ITS; at 192 hpi, embryos were removed and medium was collected (PVP 80-192 hpi) (D) (E) BSA: embryos were cultured in BSA/ITS until 192 hpi, serving as a positive control. Only PVP/ITS medium was collected for mass spectrometry.*

Blastocyst development was slower in defined medium, since fewer blastocysts could be observed at 168 hpi when embryos were cultured the whole culture period in PVP compared to BSA.

At 192 hpi, however, no differences were observed anymore for all treatments compared with the control concerning blastocyst development and hatching rate. Proteins could be identified in the spent culture medium in all three samples: 2 proteins in the group PVP till 80hpi, 16 proteins in the group PVP 80-192 hpi and 35 in the group PVP till 192 hpi (inclusion criteria: p-value of peptide <0.01 and minimal 2 sequences per protein identified) (Figure 3). In all three samples albumin was still the most abundant protein present, but we were able to identify some proteins by using defined culture medium. Based on these results, we considered another challenge is the fact that 50 to 70 % of bovine embryos cultured *in vitro* may arrest during development. Arrested or dying embryos may contain cells with a damaged cell membrane causing a passive leakage of proteins into the culture medium that might mask the actively secreted autocrine factors as well and lead to false conclusions. To this end, the embryos were cultured individually in defined embryo culture medium and additionally, the membrane integrity of these *in vitro* produced embryos was checked in order to differentiate between good quality embryos and embryos of poor quality with a high percentage of membrane-damaged cells, as described in **chapter 6**. Doing so, we have identified several possible embryotrophic proteins by means of tandem mass spectrometry using individual culture of bovine embryos in albumin-free culture medium and confirmed a critical role for Cathepsin-L on preimplantation embryo development.

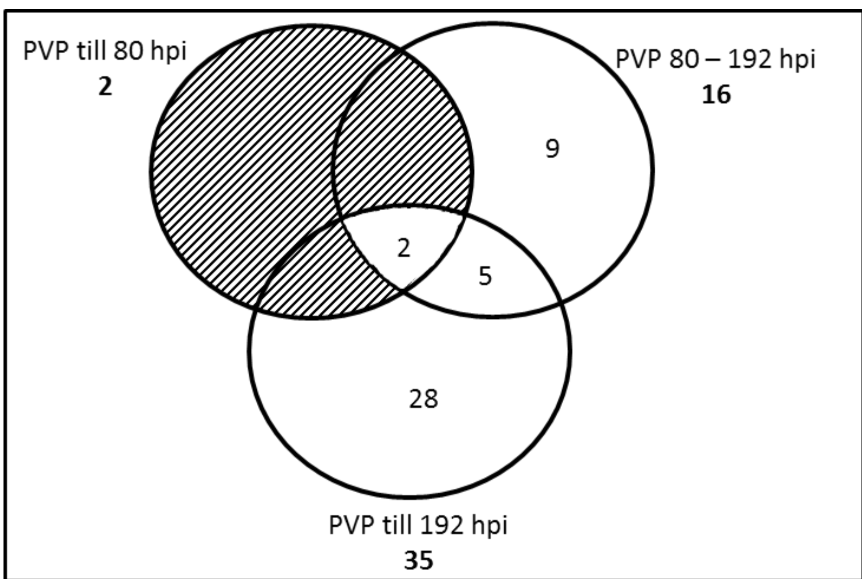


Figure 3. *Venn diagram: the numbers of proteins identified in defined culture medium, in which bovine embryos were cultivated before embryonic genome activation (PVP till 80hpi), after embryonic genome activation (PVP 80-192 hpi), or during the whole culture period (PVP till 192 hpi).*

Downside of this approach is that individual culture is not the ideal setting to study inter-embryonic communication, since no interactions between the embryos are possible. Therefore, a possible next step would be to verify if the proteins secreted by individually cultured ‘excellent’ and

'good' quality embryos, as defined in **chapter 6**, are also secreted during group culture. By using the isobaric tags for relative and absolute quantitation (iTRAQ) technology, it is possible to identify proteins present in a sample combined with a relative quantification of the proteins. Therefore, the embryo-conditioned medium of embryos cultured in group, medium conditioned by individually cultured 'excellent' and 'good' quality embryos and thirdly to medium conditioned by individually cultured 'poor' quality embryos should get a different iTRAQ label. Afterwards, the pool of these 3 groups can be subjected to LC-MS/MS: like this, the proteins present in embryo conditioned medium can be identified and the relative abundance of the identified proteins in the samples can be evaluated (Wiese, et al. 2007). As a result, the proteins present in group culture can be compared to the proteins present in medium conditioned by 'good' or 'poor' quality embryos. Another downside of the approach adopted in this thesis, is the use of defined medium since it is a general concern that albumin-free medium supports embryo development to a lesser extent than medium with albumin. Albumin is the most common protein of the female genital tract fluids, and is also a very popular supplement during *in vitro* culture of embryos. The addition of albumin to culture medium has been shown support embryo development in many different ways, e.g. by modifying oxidation of pyruvate (Eckert, et al. 1998; Lee, et al. 1998) or by contamination products of commercial BSA as citrate (Gray, et al. 1992) and an undefined factor (Sung, et al. 2004) that may act as embryotrophic factors. As discussed in **chapter 1**, albumin is also an important carrier molecule of autocrine factors, as e.g. PAF (Ammit and O'Neill 1997). Additionally, albumin may neutralize some toxins (Gardner 2008) and so having an important role in both positive and negative conditioning of culture media. Besides albumin is added to culture medium as a surfactant, preventing the embryos to adhere to plastic, and to maintain colloid osmotic pressure (Garner 2008). In chapter 6, we observed a slower embryo development both in group culture as in individual culture compared to culture in SOF with albumin. Recently, it has been shown that the quality of the obtained blastocysts in PVP medium was lower compared to BSA medium in terms of a lower total cell number and higher apoptotic cell ratio (Pavani, et al. in preparation). The medium used in these experiments was not completely free of albumin: although we started from albumin-free culture media, albumin remained the most abundant protein in all analysed samples, most probably by contamination of the HEPES-talp wash step. We strongly believe that bovine embryos need a low concentration of albumin, since further reducing the albumin concentration by using albumin-free HEPES-TALP affected embryo development in a negative way. Since it has been shown already that the composition of culture medium has a serious impact on mRNA expression of *in vitro* produced bovine embryos (Arias, et al. 2013, Heras, et al. 2016), these alterations in gene expression will also affect the secretome of the embryos. However, up till now, no data are available on the impact of defined medium on embryonic gene expression.

3. Outcome parameters

The ultimate test of the quality of an embryo is to test its ability to implant after transfer, leading to successful pregnancy and to deliver living, healthy offspring. Transferring the embryos to recipient cows would have been the golden standard to evaluate the different culture systems used in this thesis. However, this is a time-consuming and very expensive method, even more because the oocytes were collected from slaughterhouse materials, since the genetic background was not important, meaning that transfer of these embryos would be economically invaluable. Several non-invasive and invasive methods have been described to assess embryo viability indirectly or to predict the implantation potential in commercial settings. In this thesis, the different outcome parameters to assess the embryo viability were: i) the cleavage rate at 45 hpi and development to the blastocyst stage at 168 and 192 hpi, ii) the kinetics of cleavage at 45 hpi, iii) hatching rate at 192 hpi and iv) a differential apoptotic staining was performed to evaluate the total cell number, ICM ratio and apoptotic cell ratio.

3.1 Non-invasive parameters

Assessment of the developmental stage and morphology is at present the most widespread non-invasive method for bovine embryo selection, albeit imperfect (Van Soom, et al. 2003, Bavister 1995). It has been shown in cattle that there is a positive relationship between the morphological quality of embryos and the subsequent pregnancy rate (Alvarez, et al. 2008). Also in human IVF, daily embryo morphology evaluation (based on fragmentation, number of blastomeres, multi-nucleation, uneven cleavage) is the most popular tool to filter out the embryos with the highest chance to result in pregnancy (for review see Ebner, et al. 2003). However, not all suboptimal culture conditions are incompatible with normal blastocyst formation: preimplantation embryos adapt easily to the environment in which they are cultured (Lonergan, et al. 2006). Likewise, blastocysts evaluated as morphologically normal under a stereomicroscope are not necessarily viable (Hardy, et al. 1989), since *in vitro* culture conditions may have long-term effects on implantation potential and post-natal growth and development (for review see Fleming, et al. 2004).

Besides embryo development, the kinetics of the early cleavage stages were considered in this thesis. Fast and slow cleaving embryos were evaluated at a single time point: embryos that had proceeded to the third cleavage division (5–8 cells) at 45 hpi were classified as 'fast embryos' whereas those that had reached only the second cleavage division (2–4 cells) at 45 hpi were categorized as 'slow embryos'. The time points and criteria for the selection of fast or slow developing embryos have been determined on the basis of previous studies in similar culture conditions (Dinnyes, et al. 1999,

Lequarre, et al. 2003, Lonergan, et al. 1999, Vandaele, et al. 2007). However, it has been shown that recurrent examinations at successive time points can reveal important differences between embryos in the time of reaching a certain cell-stage and in the time necessary to progress from one cleavage division to the next (Grisart, et al. 1994, Gutierrez-Adan, et al. 2001, Gutierrez-Adan, et al. 2004, Holm, et al. 2002, Holm and Callesen 1998, Holm, et al. 1998, Lonergan, et al. 2000, Lonergan, et al. 1999, Van Langendonckt, et al. 1997). Timing of the first cleavage has a major influence on the probability of an embryo developing to the blastocyst stage, with fast-cleaving embryos being more likely to develop to blastocyst stage (Dinnyes, et al. 1999, Lonergan, et al. 1999). In general, fast cleaving embryos are considered more *in vivo* like, since embryo development *in vitro* is slower than that *in vivo* (Van Soom, et al. 1997b). The number of observations can be further increased by using time-lapse cinematography: a continuous imaging system to track kinetics and morphological features of *in vitro* embryo development without removal from controlled and stable incubator conditions (reviewed by Kirkegaard, et al. 2012). Time-lapse imaging of bovine embryos has revealed prognostic factors that reflect viability after transfer (Sugimura, et al. 2012, Sugimura, et al. 2017) and has as already been introduced into clinical practice in many human IVF laboratories. However, up till now conflicting data about the added value of time lapse imaging in human IVF have been published (for review see Bhide, et al. 2017, Chen, et al. 2017).

Hatching rate was assessed in this thesis as the number of hatching or hatched blastocysts compared to the total number of blastocysts formed at 192 hpi. Continuous expansion of the blastocoele is leading to progressive global thinning and finally focal rupture of the ZP. By using time-lapse systems, repeated collapses and expansion of the blastocyst have been observed prior to hatching of bovine embryos (Niimura, et al. 2010, van Heule, et al. 2001). Hatching, or the escape of the embryo from the surrounding zona pellucida (ZP), is necessary for subsequent implantation after transfer and might therefor be considered as an important indicator for viability. However, it has been shown that higher pregnancy rates after transfer to recipients could be observed compared to the observed hatching rates after prolonged *in vitro* culture at 216 hpi (Hoelker, et al. 2006). Presumably this is due to the fact that *in vitro* hatching is very different from the process *in vivo*, where a gradual zona lysis is observed independent of blastocyst expansion, and with more oval shaped blastocysts (Gonzales and Bavister 1995, Montag, et al. 2000).

Analysis of the culture medium that surrounds the embryo can be used as a non-invasive method to assess embryo viability, since it is the direct microenvironment of the IVP embryo. Measurable metabolic components in correlation with embryo viability include glucose, lactate, and pyruvate (Gardner, et al. 2001) and oxygen consumption (Lopes, et al. 2007). It has also been

demonstrated that there is a link between embryo viability and its consumption/production of amino acids (Sturmey, et al. 2009). Furthermore identification of specific markers secreted into the culture medium by *in vitro* produced embryos can be related to embryo viability. Platelet activation factor is the first molecule that has been used to evaluate the embryo viability and predict the pregnancy outcome, as the embryonic PAF level is positively correlated to the pregnancy rate (Roudebush, et al. 2002). Another possible marker of embryo developmental potential, secreted by the embryo, is soluble human leucocyte antigen-G (Desai, et al. 2006) and ubiquitin (Katz-Jaffe, et al. 2006). In bovine embryo production, no secreted marker for embryo viability has been identified up till now. The main disadvantage of these methods is that they are limited to individually cultured embryos and are therefore not useful in this thesis.

3.2 Invasive parameters

The development of a robust, reliable protocol for a differential apoptotic staining (**Chapter 3**) allowed concurrent assessment of total cell number, ICM/TE ratio and apoptotic cell ratio. All parameters can be assessed simultaneously in one single embryo, decreasing necessary sample size of the performed experiments. Although for none of these parameters a cut-off value exist to classify an embryo as good or bad quality, they are very popular parameters. Total cell number of *in vitro* produced embryos has been shown to be a valid indicator of the viability (Papaioannou and Ebert 1988). In **chapter 5**, the total cell number of the different blastocyst stages has been determined: we observed that the further the development, the higher the average total cell number, although there was a large overlap between the different stages. Furthermore, Jiang et al. (1992) reported that the total cell number was higher in bovine IVF blastocysts that received a higher morphological grade irrespective of the blastocyst stage.

Besides the total cell number, cell lineages (ICM and TE numbers) play a fundamental role in the embryo survival and foetal viability. Starting from morula stage, a segregation between the cells of the trophectoderm (TE) and the inner cell mass (ICM) occurs (Van Soom, et al. 1997a, Van Soom, et al. 1997b). Both cell lineages are essential for survival of the embryo since the ICM cells will give rise to all embryonic tissues and TE cells to the extra-embryonic membranes, which are a part of the placenta. In pigs, it has been shown that *in vitro* culture severely decreased the number of ICM cells compared to *in vivo* produced embryos (Machaty, et al. 1998). In bovine, *in vitro* produced embryos have a lower ICM/TE ratio compared to their *in vivo* counterparts (Van Soom, et al. 1997a, Van Soom, et al. 1997b). Bovine good quality *in vitro* embryos are considered to have an ICM/TE ratio of 1:3 (Maylem, et al. 2017), representing an ICM/TCN ratio, as is calculated in this thesis, of 1:4. Microscopic

scoring of the size of the ICM can be used as a rough estimate of the number of ICM cells, however a differential staining remains necessary to evaluate the proportion ICM/TCN cells. In this thesis, the difference between ICM and TE cells was based on the unique expression of the transcription factor CDX-2 in the TE cells. The presence of CDX-2 was visualized by indirect fluorescent labelling with Texas Red secondary antibodies. Unfortunately, Hoechst, which was used to stain all nuclei of the blastocyst, and Texas Red cannot be evaluated using the same filter cube of the fluorescent microscope (Figure 4). The reason for this is because Hoechst and Texas Red do not have the same excitation-emission spectra. This is different from the earlier described protocols, using a combination of Hoechst and Propidium iodide, that can be visualized together using the correct long pass filter on the fluorescent microscope. As a consequence, the number of ICM cells cannot be counted directly, but had to be calculated as TCN (Hoechst positive cells) – TE (Texas Red positive cells). This can be circumvented by selecting a marker specific for ICM cells instead of TE cells. OCT4, NANOG, and SOX2 are transcription factors exclusively expressed in the inner cell mass of mouse blastocysts (Avilion, et al. 2003, Chambers, et al. 2003, Scholer, et al. 1990). In contrast, in bovine blastocysts, OCT4 and NANOG can be detected in both ICM and TE cells (Kirchhof, et al. 2000, Munoz, et al. 2008). Recently, SOX2 has been shown to be a specific marker for ICM-cells of bovine embryos (Goissis and Cibelli 2014) and a differential staining protocol has been optimized based on SOX2 (Moreno, et al. 2015).

In parallel with the TCN and ICM/TCN ratio, the apoptotic cell ratio can be evaluated in a single embryo. For a long time, it was believed that the triggers for apoptosis were mainly endogenous in origin (‘cellular suicide’). However, a suboptimal culture environment is thought to be an important exogenous trigger for apoptosis. This is affirmed by the lower degree of apoptosis in *in vivo* embryos in comparison with *in vitro* embryos, especially in the ICM (Brison and Schultz 1997, Gjorret, et al. 2003, Levy, et al. 2001) and the apoptotic cell ratio increased by increasing duration of the culture period (Vandaele, et al. 2006). By using a differential apoptotic staining, a lot of data can be obtained about the quality of one blastocyst. However, more information could be collected if the parameters would have been assessed for each blastocyst stage (young – normal – expanded and hatching/hatched on 8dpi) seperately. In addition, the apoptotic cells ratio could have been calculated for ICM and TE cells separately. During evaluation of the staining, we had the impression that apoptosis predominantly occurred in the ICM.

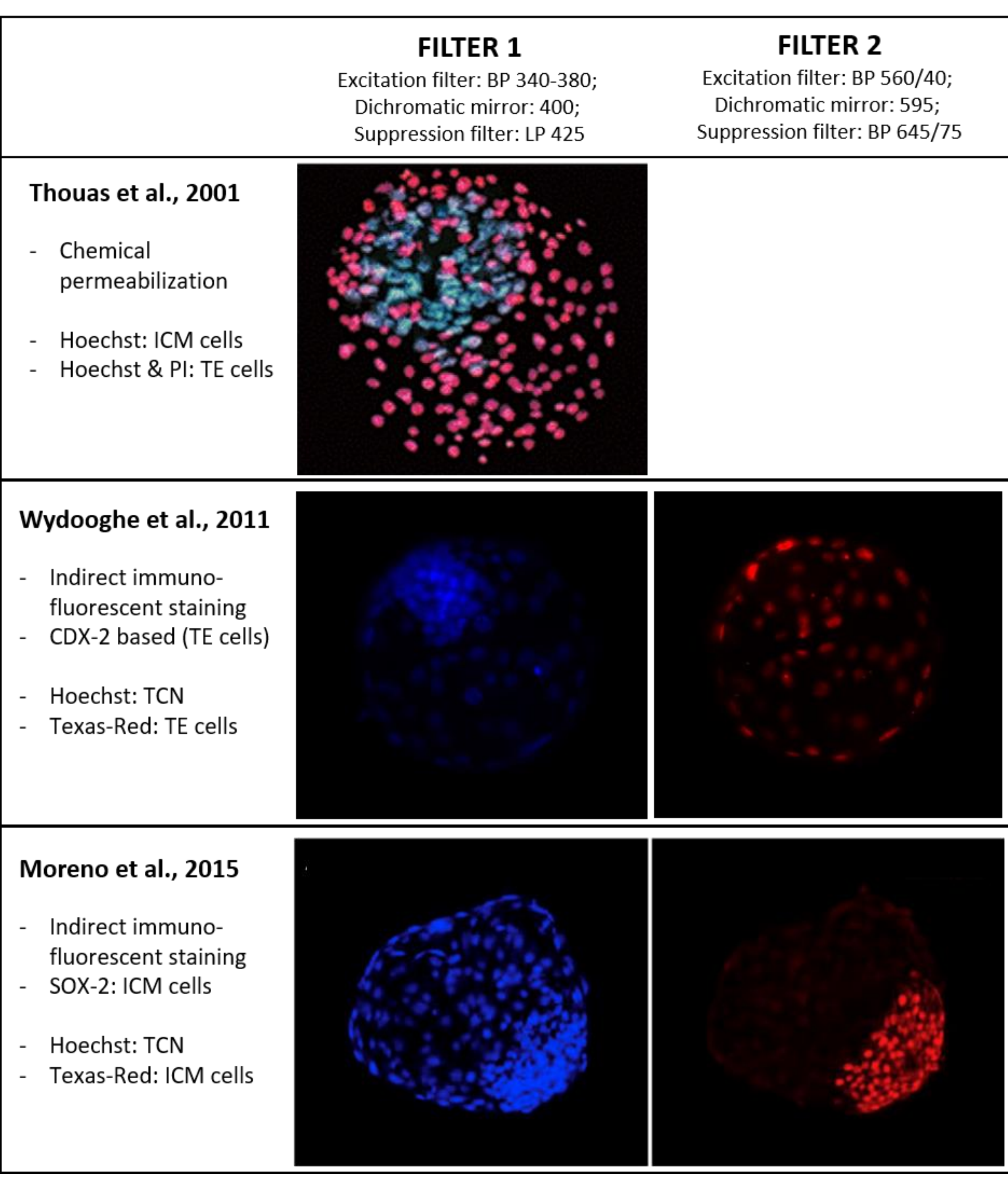


Figure 4: *Interpretation of the different protocols for differential staining of bovine blastocysts.*

Preimplantation embryos possess an enormous degree of flexibility, which may lead to aberrations in the gene expression pattern of *in vitro* produced embryos (reviewed by Hyttel, et al. 2000). Compared to *in vivo* derived embryos, genes related to stress were up-regulated in IVP embryos, whereas genes related to metabolism, growth and differentiation were up-regulated in *in vivo* embryos (Gutierrez-Adan, et al. 2004, Lazzari, et al. 2002, Rizos, et al. 2003). Furthermore, it has also been shown that culture media composition, but also the culture conditions as oxygen concentration may affect the gene expression patterns of the obtained embryos (For review see Gad, et al. 2012).

Recently, it has been shown that embryos cultured in group in SOFaa supplemented with BSA and ITS showed gene expression patterns that were more similar to those derived *in vivo* than embryos produced in serum containing culture medium (Heras, et al. 2016). These alterations in gene expression are thought to be induced by epigenetic modulations, most likely by changes in the methylation pattern. Epigenome is able to regulate the expression of genes without changing the DNA sequence, enabling select genes to be active at different times. Earlier, these epigenetic modifications have been considered to be static modifications, however lately, it has demonstrated that epigenetic modifications of the genome are a lot more dynamic than initially thought. The epigenome appears to be most flexible during early embryo development, changing in each cell lineage during development, and this steadily decreases during prenatal and postnatal life. Epigenetic changes can be transient or persistent influencing disease susceptibilities later in life (El Hajj and Haaf 2013). Large offspring syndrome (LOS) is a well-known example of a syndrome related bovine and ovine *in vitro* embryo production. The features of LOS include: excessive birth weight, large tongue, breathing difficulties, reluctance to suckle and sudden perinatal death (Young, et al. 1998). This syndrome has been ascribed to a reduced methylation in the imprinted IGF-2R gene, leading to reduced expression (Young, et al. 2001). Recent studies showed that other epigenetic alterations as hypomethylation of the KvDMR1 and bi-allelic expression of KCNQ1OT1 can also be linked to LOS (Couldrey and Lee 2010, Hori, et al. 2010). In humans, the absolute risk in babies born through ART to develop a disorder caused by epigenetic alterations, such as Beckwith-Wiedemann syndrome and Angelman, is low, however the relative risk when compared to non-ART babies is significantly higher (reviewed by Allen and Reardon 2005). How the culture systems and the addition of the identified proteins in this theses affect the gene expression and epigenetic patterns of the obtained embryos, definitely remains to be elucidated further.

4. Conclusions and outlook

- Up till now, individual culture of bovine embryos has been associated with low blastocyst development. However, replacing serum by bovine serum albumin (BSA) and insulin, transferrin and selenium (ITS) is supporting embryo development in individual culture to the same extent as group culture. Nevertheless, group culture still improves blastocyst quality.

- The development of a semi-defined individual culture system is not only an important tool for research laboratories and practitioners working with bovine IVF, in addition the value of the bovine embryos as a model for human IVF was upgraded. However, it is important to further improve this

culture system so that the quality of the obtained blastocysts reaches the same level or higher than that of embryos cultured in groups.

- In order to do so, commercially available systems allowing group culture and individual follow-up were tested using bovine embryos. Primo Vision® dishes provide an excellent system that combines the benefits of group and individual culture, leads to a similar rate of embryo development compared to classical group culture and gives better embryo development and quality than individual culture or culture in Corral® dishes.

- Using the Primo Vision® dishes, we showed that the net outcome of group culture of bovine embryos in Primo Vision® dishes is advantageous compared to individual culture, giving a higher blastocyst development rate and improved blastocyst quality. This was obtained because more slow cleaving embryos reach the blastocyst stage in group culture. In general, non-cleaved or arrested embryos did not negatively affect the development of the surrounding embryos. However, care should be taken when 'fast' embryos are group cultured and only a few of the surrounding embryos reach the blastocyst stage since this could compromise blastocyst development compared to individually cultured 'fast' embryos.

- Although the promising results obtained for bovine embryos in Primo Vision® dishes, this could not be confirmed for human embryos in the interim analysis of the multicentre clinical randomized trial.

- An easy and repeatable method for differential staining of the obtained blastocysts was developed, in order to evaluate the quality of the obtained bovine embryos in the different group culture systems tested. Likewise, we could evaluate three parameters at once: total cell number (TCN), ICM-ratio, and the apoptotic cell ratio (ACR). Although these are valuable outcome parameters, further research is needed to gain more insight in the viability of the obtained blastocysts, preferably with a transfer study.

- Proteomic analysis of spent bovine embryo culture medium necessitates the use of individual culture in albumin-free culture medium in combination with a fluorescent staining to evaluate membrane integrity of the blastomeres. We were able to identify proteins that were secreted by bovine embryos during the preimplantation period into the culture medium. Addition of Cathepsin-L to the culture medium, confirmed embryotrophic properties of Cathepsin-L during bovine preimplantation *in vitro* embryo development. Further research is required to compare the secretome of group cultured embryos with individually cultured embryos. Preferably culture medium supplemented with albumin should be analysed, since the lack of albumin has an influence

on embryo development and blastocyst quality, most probably affecting the secretome of the embryos.

- By proteomic analysis we could identify proteins secreted into the culture medium by bovine embryos. More research is needed to evaluate the effect of all these identified proteins. Furthermore, the optimal way of supplementing these proteins to the culture medium should be evaluated. For example, it might be interesting to add the proteins encapsulated in exosome-like structures to prevent degradation in the culture medium and as such, increasing their accessibility to the embryos.

- Inter-embryonic communication is very complex: embryotrophic factors with different biochemical characteristics can be secreted by different mechanisms and act through pathways. This thesis did not solve the puzzle of inter-embryonic communication during group culture, but provided the first piece of the puzzle to further optimize *in vitro* embryo culture conditions, both for human and bovine embryos, based on these embryotrophic factors.

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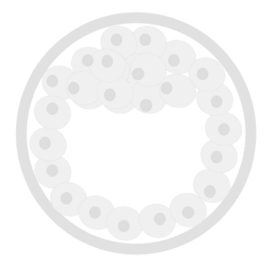


CHAPTER 8

SUMMARY

SAMENVATTING

SUMMARY

In vitro production of embryos (IVP) has evolved immensely since the birth of the first test-tube baby, Louise Brown, in 1978. In humans, the main goal of IVP is to bypass reproductive failure and is in this respect totally different from IVP in animals, where it is mainly performed to improve genetics and more recently also to conserve endangered species. Furthermore, in research labs large numbers of bovine IVP embryos are produced that can increase our understanding of basic biological processes and can be used as a model for human embryos. Embryo production *in vitro*, implies an extraordinary change in the environment for maturation and fertilisation of the oocytes and the first cleavages of the embryos, compared to the *in vivo* situation. During *in vivo* development, a specific communication between the mother and embryo is necessary, in order to create the optimal environment for early embryo development, implantation, maintenance of pregnancy and the future health of the offspring. This communication *in vivo* is established by a combination of autocrine, paracrine and endocrine factors. During *in vitro* embryo production all this communication with the maternal tract is cut off and only the autocrine factors remain. Autocrine factors are factors released by the embryo itself and act upon the embryo or the neighbouring embryos. These autocrine factors can be very diverse in origin and act through different pathways, as discussed in **chapter 1**. The presence of autocrine factors is believed to be the reason why embryos cultured in group thrive better than embryos cultured individually. Group culture has been adopted in many mammalian IVP labs, however in human IVP, embryos are standardly cultured individually. The aim of this thesis was to investigate and identify how inter-embryonic communication of bovine embryos occurs when cultured in group. The obtained knowledge can be used to further optimise the culture system to produce human embryos *in vitro*. As discussed in **chapter 1**, bovine embryos represent an attractive model for human studies.

First, we established an new protocol for differential apoptotic staining, in order to be able to evaluate the quality of the obtained bovine blastocysts. **Chapter 3** describes the protocol for a double indirect immunofluorescent staining in order to be able to count the total cell number, the inner cell-mass ratio and the apoptotic cell ratio in one embryo all at once. The differential staining is based on the transcription factor CDX2, which is localized in the nucleus of trophectoderm (TE) cells, but absent in the inner cell mass (ICM). Apoptosis is detected by staining of active caspase-3, a key player in

several apoptotic pathways. Concurrently, all nuclei are stained with the DNA stain Hoechst 33342; in order to be able to count the total cell number. The current staining has been proven to be equally successful in human, murine and porcine embryos.

Individual culture of bovine embryos was for a long time leading to very disappointing results. However, in **chapter 4**, we observed high blastocyst development after individual embryo culture in a serum-free culture system, based on bovine serum albumin (BSA) and insulin, transferrin and selenium (ITS). We therefore hypothesized that serum has a negative effect on embryos cultured individually, whereas embryos in groups can counteract this. The development of an individual culture system with repeatable high blastocyst percentages, upgrades the value of bovine embryos as a model for human IVF. Importantly, although serum-free individual culture is leading to similar blastocyst rates compared to group culture, the quality of the obtained blastocysts remains inferior in terms of lower total cell numbers and more apoptosis.

In **chapter 5**, Corral® and Primo Vision® dishes, two group culture devices that could potentially be used to culture human embryos were tested. These two culture devices combine the benefits of group culture with the possibility to track the development of each embryo separately during the whole culture period. We tested the devices in a bovine setting and were able to show that blastocyst development in Primo Vision® dishes was similar to classical group culture and better than in Corral® dishes or individual culture. Furthermore, we obtained more insights in the group culture phenomenon, since we could show that in the Primo Vision® dishes, a higher number of 'slow' embryos developed to the blastocyst stage compared to their individually cultured counterparts, while no differences were observed for 'fast' embryos. 'Slow' embryos in a 'standard drop' had a higher chance of becoming a blastocyst compared to individual culture, whereas blastulation of 'fast' embryos was less efficient in a 'delayed drop' than in individual culture. Furthermore, we could show that non-cleaved or arrested embryos do not hamper the ability of co-cultured bovine embryos to reach the blastocyst stage in group culture.

The identity of the autocrine factors responsible for the improved embryo development in group culture remains for the greater part unknown. In **chapter 6**, we used a screening wide proteomic approach so that the proteins that are secreted into the culture medium by preimplantation bovine embryos can be identified. To this end, bovine embryos had to be cultured individually in albumin-free embryo culture medium and additionally, the membrane integrity of these *in vitro* produced embryos was checked in order to differentiate between good quality embryos and embryos of poor quality with a high percentage of membrane-damaged cells. In the spent culture medium of blastocysts with no membrane damaged cells, Cathepsin-L could be identified as a possible autocrine factor actively

secreted into the medium. Addition of Cathepsin-L to the culture medium of individually cultured embryos was able to progress the first cell divisions, and improve both blastocyst development as well as blastocyst quality improved compared to individual culture in the absence of Cathepsin-L. The identification of potential autocrine factors and the observed positive effect of the addition to the culture medium, is a promising approach to optimise the culture media used for both bovine and human *in vitro* embryo production.

The general discussion and the conclusions of this thesis are presented in **chapter 7**:

- Up till now, individual culture of bovine embryos has been associated with low blastocyst development. However, replacing serum by bovine serum albumin (BSA) and insulin, transferrin and selenium (ITS) is supporting embryo development in individual culture to the same extent as group culture. Nevertheless, group culture still improves blastocyst quality.
- The development of a semi-defined individual culture system is not only an important tool for research laboratories and practitioners working with bovine IVF, in addition the value of the bovine embryos as a model for human IVF was upgraded. However, it is important to further improve this culture system so that the quality of the obtained blastocysts reaches the same level or higher than that of embryos cultured in groups.
- In order to do so, commercially available systems allowing group culture and individual follow-up were tested using bovine embryos. Primo Vision® dishes provide an excellent system that combines the benefits of group and individual culture, leads to a similar rate of embryo development compared to classical group culture and gives better embryo development and quality than individual culture or culture in Corral® dishes.
- Using the Primo Vision® dishes, we showed that the net outcome of group culture of bovine embryos in Primo Vision® dishes is advantageous compared to individual culture, giving a higher blastocyst development rate and improved blastocyst quality. This was obtained because more slow cleaving embryos reach the blastocyst stage in group culture. In general, non-cleaved or arrested embryos did not negatively affect the development of the surrounding embryos. However, care should be taken when 'fast' embryos are group cultured and only a few of the surrounding embryos reach the blastocyst stage since this could compromise blastocyst development compared to individually cultured 'fast' embryos.
- Despite the promising results obtained for bovine embryos in Primo Vision® dishes, this could not be confirmed for human embryos in the interim analysis of the multicentre clinical randomized trial.

- An easy and repeatable method for differential apoptotic staining of the obtained blastocysts was developed, in order to evaluate the quality of the obtained bovine embryos in the different culture systems tested. Likewise, we could evaluate three parameters at once: total cell number (TCN), ICM-ratio, and the apoptotic cell ratio (ACR). Although these are valuable outcome parameters, further research is needed to gain more insights in the viability of the obtained blastocysts, preferably with a transfer study.
- Proteomic analysis of spent bovine embryo culture medium necessitates the use of individual culture in albumin-free culture medium in combination with a fluorescent staining to evaluate membrane integrity of the blastomeres. We were able to identify proteins that were secreted by bovine embryos during the preimplantation period into the culture medium. Addition of Cathepsin-L to the culture medium, confirmed embryotrophic properties of Cathepsin-L during bovine preimplantation *in vitro* embryo development. Further research is required to compare the secretome of group cultured embryos with individually cultured embryos. Preferably culture medium supplemented with albumin should be analysed, since the lack of albumin has an influence on embryo development and blastocyst quality, most probably affecting the secretome of the embryos.
- By proteomic analysis we could identify proteins secreted into the culture medium by bovine embryos. More research is needed to evaluate the effect of all these identified proteins. Furthermore, the optimal way of supplementing these proteins to the culture medium should be evaluated. For example, it might be interesting to add the proteins encapsulated in exosome-like structures to prevent degradation in the culture medium and as such, increasing their accessibility to the embryos.
- Inter-embryonic communication is very complex: embryotrophic factors with different biochemical characteristics can be secreted by different mechanisms and act through different pathways. This thesis did not solve the puzzle of inter-embryonic communication during group culture, but provided the first piece of the puzzle to further optimize *in vitro* embryo culture conditions, both for human and bovine embryos, based on these embryotrophic factors.

SAMENVATTING

De eerste proefbuisbaby, Louise Brown, werd in 1978 geboren, en sindsdien werden de technieken voor *in vitro* productie (IVP) van embryo's verder ontwikkeld en verfijnd. *In vitro* productie van embryo's is bij mensen een belangrijke behandeling bij een onvervulde kinderwens. Dit staat in groot contrast tot IVP bij dieren, waarbij de techniek voornamelijk ingezet wordt voor economische of foktechnische redenen. Recent wordt de techniek ook aangewend voor het behoud van bedreigde diersoorten. Daarnaast worden in onderzoekslaboratoria grote aantallen runderembryo's geproduceerd die kunnen bijdragen aan onze kennis over biologische processen en kunnen gebruikt worden als model voor humane embryo's. Wanneer embryo's *in vitro* geproduceerd worden, betekent dit dat de eicelrijping, de bevruchting en de eerste celdelingen in een totaal andere omgeving moeten plaatsvinden dan in de *in vivo* situatie. Tijdens de *in vivo* ontwikkeling is er een specifieke communicatie tussen moeder en embryo nodig om een ideale omgeving te creëren voor de vroege embryonale ontwikkeling, de implantatie, het behoud van de dracht of zwangerschap en de toekomstige gezondheid van de nakomeling(en). Deze communicatie komt tot stand door een combinatie van autocriene, paracriene en endocriene factoren. Wanneer embryo's in het labo geproduceerd worden valt deze communicatie helemaal weg, met uitzondering van de autocriene factoren. Autocriene factoren worden vrijgesteld door een embryo en hebben een effect op het embryo zelf of op de naburige embryo's. In **hoofdstuk 1** worden de verschillende soorten autocriene factoren besproken, alsook de verschillende pathways waarop deze factoren kunnen inwerken. Er wordt algemeen aangenomen dat de aanwezigheid van deze autocriene factoren in een groepscultuursysteem, aan de basis liggen van de betere ontwikkeling ten opzichte van individuele cultuur van embryo's. De algemene doelstelling van deze studie is om de manier waarop de communicatie tussen embryo's in een groepscultuursysteem verloopt te onderzoeken en te identificeren. De verkregen kennis kan gebruikt worden om het cultuursysteem voor de *in vitro* productie van humane embryo's verder te verbeteren. Zoals besproken in **hoofdstuk 1**, vormen runderembryo's een ideaal model voor humane studies.

In eerste instantie hebben we een nieuw protocol voor een differentiële apoptotische kleuring geoptimaliseerd zodat de kwaliteit van de verkregen runderembryo's kan geëvalueerd worden. In **hoofdstuk 3** wordt het protocol voor een dubbele indirecte immunofluorescente kleuring beschreven

waarmee het mogelijk is om het totaal celaantal, de verhouding van het aantal kiemknopcellen tot het totaal celaantal en de verhouding van het aantal apoptotische cellen tot het totaal celaantal te berekenen. De differentiële kleuring is gebaseerd op de aanwezigheid van de transcriptiefactor CDX2, die enkel tot uiting komt in de kern van de trofectodermcellen, maar niet in de kiemknopcellen. Apoptose wordt gedetecteerd door de aanwezigheid van actief caspase-3, die een belangrijke rol speelt in verschillende apoptotische pathways. Tegelijk worden alle kernen aangekleurd met Hoechst 33342, een kleurstof die bindt op DNA, zodat het totaal aantal cellen kan geteld worden. De beschreven techniek werd ook succesvol toegepast om de kwaliteit van embryo's van mens, muis en varken te evalueren.

Tot nu toe zijn de beschreven resultaten na individuele cultuur van runderembryo's teleurstellend. In **hoofdstuk 4** konden we hoge percentages blastocysten bekomen na individuele cultuur in een serum-vrij cultuursysteem, gebaseerd op bovien serum albumine (BSA) en insuline, transferrine en selenium (ITS). Bijgevolg was onze hypothese dat serum een negatief effect heeft op de ontwikkeling van embryo's die individueel gecultiveerd worden, daar waar embryo's die in groep gecultiveerd worden opgewassen zijn tegen dit negatieve effect. Het ontwikkelen van een individueel cultuursysteem dat leidt tot herhaalbare, hoge blastocystpercentages verhoogt nog verder de waarde van runderembryo's als model voor humane IVP. Een belangrijke bevinding is dat de kwantitatieve ontwikkeling tot blastocyst niet verschilt tussen individuele cultuur en groepscultuur van runderembryo's in serum-vrij medium, maar wel de kwaliteit van de verkregen blastocysten.

In **hoofdstuk 5** worden twee commercieel beschikbare groepscultuursystemen uitgetest, die kunnen gebruikt worden om humane embryo's te cultiveren: Corral® en Primo Vision® plaatjes. Deze twee systemen combineren de voordelen van groepscultuur met de mogelijkheid om de ontwikkeling van elk embryo afzonderlijk op te volgen. In een test met runderembryo's konden we aantonen dat de ontwikkeling tot het blastocyststadium in Primo Vision® plaatjes vergelijkbaar was met het klassieke groepscultuursysteem en tegelijk beter dan de ontwikkeling in Corral® plaatjes en in individuele cultuur. We verkregen ook meer inzicht in het groepscultuur fenomeen: in de Primo Vision® plaatjes bereiken meer 'trage' embryo's het blastocyststadium in vergelijking met hun individueel gecultiveerde tegenhangers, voor de 'snelle' embryo's merken we geen verschil op. 'Trage' embryo's die in een 'standaard druppel' gecultiveerd worden hebben een grotere kans om het blastocyst stadium te bereiken in vergelijking met individuele cultuur. Voor 'snelle' embryo's werd er een negatief effect opgemerkt wanneer zij in een 'minder goede druppel' gecultiveerd werden. Daarnaast konden we ook aantonen dat niet-gedeelde embryo's de ontwikkeling van de andere embryo's in een groepscultuursysteem niet hinderen.

De identiteit van de autocriene factoren die verantwoordelijk zijn voor de betere embryonale ontwikkeling in een groepscultuursysteem is nog grotendeels onbekend. In **hoofdstuk 6** werden de eiwitten, vrijgesteld in het cultuurmedium door runderembryo's, geïdentificeerd met behulp van proteoomanalyse. Hiervoor werden runderembryo's individueel gecultiveerd in albumine-vrij medium en bijkomend werd de membraanintegriteit getest om de embryo's van goede kwaliteit te kunnen differentiëren van de embryo's van slechte kwaliteit, die een hoog percentage membraan beschadigde cellen bezitten. Cathepsine-L is aanwezig in het cultuurmedium van blastocysten zonder membraanbeschadigde cellen en komt bijgevolg in aanmerking als autocriene factor met een positief effect. Toevoegen van Cathepsine-L aan het cultuurmedium van individueel gecultiveerde runderembryo's versnelde de eerste celdelingen en verbeterde zowel de ontwikkeling als de kwaliteit van blastocysten in vergelijking met individuele cultuur in afwezigheid van Cathepsine-L. Het identificeren van potentiële autocriene factoren en deze gebruiken als verrijking voor het cultuurmedium is een veelbelovende werkwijze om de resultaten in boviene en humane *in vitro* embryo productie te verbeteren.

De algemene discussie en de besluiten van deze studie worden voorgesteld in **hoofdstuk 7**:

- Tot op heden werd individuele cultuur geassocieerd met een verminderde blastocyst ontwikkeling. Echter, door het vervangen van serum door bovien serum albumine (BSA) en insuline, transferrine en selenium (ITS) kunnen na individuele cultuur vergelijkbare resultaten verkregen worden als na groepscultuur wat betreft de embryonale ontwikkeling. Echter, na groepscultuur wordt een betere kwaliteit van de blastocysten opgemerkt.
- Het ontwikkelen van een serum-vrij individueel cultuursysteem is niet alleen belangrijk voor onderzoekslaboratoria en deskundigen werkzaam in runder-IVP-centra, het verhoogt ook de waarde van runderembryo's als model voor humane IVF. Het is echter wel belangrijk om dit cultuursysteem verder te optimaliseren zodat de kwaliteit van de verkregen blastocysten vergelijkbaar wordt met die van embryo's die in groep gecultiveerd worden.
- Twee commercieel beschikbare cultuur systemen werden getest waarin embryo's die in groep gecultiveerd worden toch individueel kunnen opgevolgd worden. Hieruit bleek dat Primo Vision® plaatjes het best de voordelen van individuele en groepscultuur combineren: de embryonale ontwikkeling was vergelijkbaar met het klassiek groepscultuursysteem, maar beter dan individuele cultuur of cultuur in Corral® plaatjes.
- Door gebruik te maken van de Primo Vision® plaatjes konden we aantonen dat het netto positieve effect van groepscultuur van runderembryo's te wijten is aan een betere ontwikkeling van 'trage' embryo's in een groepscultuursysteem dan in individuele cultuur. Niet gedeelde embryo's

hebben geen negatief effect op de ontwikkeling van de omliggende embryo's. Wanneer echter slechts enkele van de omliggende embryo's het blastocyststadium bereiken, zullen minder 'snel' delende embryo's tot blastocyst ontwikkelen in vergelijking met individueel gecultiveerde 'snel' delende embryo's.

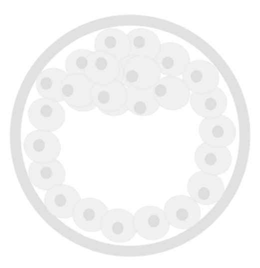
- Hoewel de resultaten van het cultiveren van runderembryo's in Primo Vision® plaatjes veelbelovend waren, kon dit niet bevestigd worden in de tussentijdse analyse van een klinische proef met humane embryo's.

- Er werd een gemakkelijke en herhaalbare methode voor een differentiële apoptotische kleuring ontwikkeld zodat de kwaliteit van de verkregen blastocysten in de verschillende cultuursystemen kan vergeleken worden. Op die manier kunnen 3 parameters tegelijk beoordeeld worden: totaal celaantal, de verhouding van het aantal kiemknopcellen tot het totaal celaantal en de verhouding van het aantal apoptotische cellen tot het totaal celaantal. Hoewel dit belangrijke parameters zijn, zou een transfer studie nog meer inzicht brengen in de kwaliteit van de verkregen blastocysten.

- Om proteoomanalyse van het medium, waarin runderembryo's werden gecultiveerd, mogelijk te maken is het nodig om een individueel albumine-vrij cultuursysteem te gebruiken gecombineerd met een fluorescente kleuring om de membraan integriteit van de blastomeren in te schatten. Op die manier kunnen eiwitten die actief gesecreteerd worden in het omliggende cultuurmedium geïdentificeerd worden. Cathepsine-L werd in deze studie geïdentificeerd als een autocriene factor. Verder onderzoek is nodig om het secretoom van individueel gecultiveerde embryo's te vergelijken met dat van embryo's die in groep gecultiveerd worden. Dit gebeurt idealiter in cultuurmedium waar albumine aan toegevoegd werd, want een gebrek aan albumine heeft een invloed op de embryonale ontwikkeling en blastocyst-kwaliteit en beïnvloedt naar alle waarschijnlijkheid het secretoom van de embryo's.

- Met behulp van proteoomanalyse konden we verschillende eiwitten identificeren die gesecreteerd worden door runderembryo's. Verder onderzoek is nodig om het effect van deze andere eiwitten te onderzoeken. Daarnaast moet ook nog de ideale methode gevonden worden om de eiwitten toe te voegen aan het cultuurmedium. Zo kan het misschien interessant zijn om de eiwitten in te kapselen in exosoom-achtige structuren voor ze toegevoegd worden aan het medium om ze op die manier toegankelijker te maken voor de embryo's.

- Communicatie tussen embryo's in een groepscultuursysteem is een complex gegeven: embryotrofe factoren met een verschillende biochemische samenstelling kunnen op verschillende manieren gesecreteerd worden en inwerken op verschillende pathways. Bijgevolg kon deze studie deze communicatie niet volledig ontrafelen, maar door deze studie werd een belangrijke eerste stap gezet voor de verdere optimalisatie van de humane en boviene *in vitro* cultuursystemen.



CURRICULUM VITAE

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Eline Wydooghe werd geboren op 18 december 1985 te Roeselare. Na het behalen van het diploma hoger secundair onderwijs aan het Bisschoppelijk Lyceum der Grauwe Zusters te Roeselare (Wetenschappen-Wiskunde), begon zij in 2003 met de studie Diergeneeskunde aan de Universiteit Gent. Zij behaalde haar diploma van Dierenarts (optie kleine huisdieren) in 2009 met onderscheiding.

Op 1 oktober 2009 trad zij als Dehousse-bursaal in dienst aan de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. Op 1 januari 2011 werd zij doctoraatsbursaal van Agentschap voor Innovatie door Wetenschap en Technologie waar zij gedurende 4 jaar onderzoek heeft gedaan naar de communicatie tussen *in vitro* geproduceerde runderembryo's in groepscultuur. Naast haar onderzoek was Eline Wydooghe ook actief in de Kliniek voortplanting gezelschapsdieren, waar ze patiënten behandelt en de studenten van het 5de en 6de jaar diergeneeskunde begeleidt tijdens hun kliniekweek. In 2011 startte ze ook als resident van het European College of Animal Reproduction (ECAR), waarvan ze in november 2015 het diploma (Subspecialiteit gezelschapsdieren) behaald heeft. In 2015 werd ze aangesteld als assistent aan de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde.

Eline Wydooghe is auteur of mede-auteur van 17 publicaties gepubliceerd in internationale en nationale wetenschappelijke tijdschriften, niet enkel publicaties over *in vitro* embryo productie, maar ook case reports en onderzoek over voortplanting kleine huisdieren. Ze was spreker op verschillende nationale en internationale congressen.

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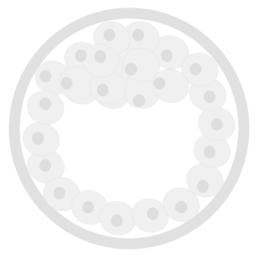

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DANKWOORD

AND IN THE END... IT ENDS ...

Finally! Terwijl jullie (of velen onder jullie ☺) dit lezen, ben ik bezig met mijn openbare verdediging: het laatste puzzelstukje van mijn doctoraat. Ik kan het maken van het doctoraat best vergelijken met het maken van een 'destiny puzzel' (een leuke winterhobby), waarbij op het deksel van de doos niet de tekening staat die je moet maken, maar wel de context en een paar verdoken hints. Gelukkig vormden sommigen onder jullie de hoekstenen of de zijkantjes van de puzzel, waarop ik verder kon bouwen. Anderen zie ik als de leuke kleuren of herkenbare figuren op de puzzel, die het onderzoek wat gemakkelijker en/of aangenamer maakte. Zonder hulp, advies en steun van de vele mensen rondom mij, was dit zeker niet gelukt. Dit is het ideale moment om iedereen te bedanken.

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