In The Name Of

God

Campus of Agriculture and Natural Resources
Department of Animal Science

M.Sc. Thesis

Title of thesis:

Comparative study of the effects of ovine, bovine and human umbilical cord blood serum on in vitro maturation and subsequent embryo development of sheep oocytes

Supervisor:

Dr. Hamed Karami Shabankareh

By:

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April 2010

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In these recounts I may have not thanked certain people. This does not mean that I am ungrateful to them; it just means that I have a lousy memory.

Dated: April, 2010 *Maryam Seidi*

Dedicated To:

My Mother,

My father,

My brother

And my husband

Abstract

The objective of this study was to investigate the influence of exogenous addition of umbilical cord serum (UCS) to maturation medium of sheep cumulus-oocyte complexes (COCs) on their cleavage and subsequent development during in vitro maturation (IVM). Sheep ovaries collected from a local abattoir were transported to the laboratory in warm phosphate-buffered saline (PBS) containing antibiotics within 2–3 h after collection. COCs were obtained by aspiration and selected by stereomicroscope. COCs were washed three times in washing medium and randomly divided into eight treatments for IVM. treatment1: COCs were cultured in maturation medium supplemented with 10% (v/v) ovine umbilical cord serum(OUCS), treatment2: COCs were cultured in maturation medium supplemented with 20%(v/v) OUCS, treatment3: COCs were cultured in maturation medium supplemented with 10% (v/v) bovine umbilical cord serum (BUCS), treatment4: COCs were cultured in maturation medium supplemented with 20% (v/v) BUCS, treatment5: COCs were cultured in maturation medium supplemented with 10% (v/v) human umbilical cord serum (HUCS), treatment6: COCs were cultured in maturation medium supplemented with 20% (v/v) HUCS, treatment7: COCs were cultured in maturation medium supplemented with %10 (v/v) fetal calf serum (FCS) as control treatment1 . treatment8: COCs were cultured in maturation medium supplemented with 20% (v/v) FCS as control treatment2. The maturation medium was TCM199 medium supplemented with 50 µg/ml gentamycin sulphate, 10µg/ml FSH, 10µg/ml LH, 1 µg/ml 17β-estradiol, 0.23 mM Na pyruvate and 50ng/ml EGF. After IVM, maturation of ovine oocyte was evaluated by cleavage and development for 6-8 days. The highest percentage of the cleavage rate was observed in the treatment7 (67.3 %) and treatment2 (65.7%) and the lowest percentage was observed in the treatment1 (44%), treatment3 (43.8%) and treatment5 (45.4%) ($p<0.05$). The morulla rate significantly increased in treatment1 (55.3%), treatment5 (54.7%) and treatment7 (61.5%) versus treatment4 (38.8%) ($p<0.05$). The blastocyst rate significantly increased in treatment7 (29.5%) and treatment۱ (27.1%) versus treatment۴ (18.2%) ($p<0.05$).

Results of the tests showed that embryos development was higher in 10% FCS but OUCS and BUCS can be support in vitro maturation of sheep oocytes.

In conclusion, IVM medium supplemented with UCS is beneficial to oocyte nuclear and cytoplasmic maturation evidenced by oocyte maturation and subsequent embryonic development. Therefore, UCS can be used as a supplement for IVM medium that may directly affect IVM outcome.

Key words: ovine, oocyte, umbilical cord serum, maturation.

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Chapter 1

Introduction

Using in vitro production of embryos allows us to obtain a large number of offspring from live or slaughtered animals (Guler et al., 2000) for research, genetic improvement or commercial purposes (Accardo et al., 2004). In sheep, techniques for in vitro fertilization and in vitro culture of embryos (IVC) seem to be under control. However in most domestic species the in vitro maturation (IVM) of oocytes needs to be perfected (Guler et al., 2000). During IVM, oocytes undergo a series of cytoplasmic changes before the resumption of nuclear maturation, leading to variable competence of the resulting embryo. The alteration of basic maturation conditions can significantly affect oocyte competence as reflected by the morula and blastocyst yield after in vitro fertilization (IVF) (Ali and Sirard, 2002). Consistently successful and reliable oocyte maturation (both cytoplasmic and nuclear maturation) would dramatically improve the efficiency of preimplantation embryonic development as well as fetal development (Sagirkaya et al., 2007).

Sera are usually added to the IVM media at the rate of 10–20% and are known to contain hormones, trace elements, growth factors and other unidentified components, some of which may have adverse effects on the development of oocytes in vitro (Purohit et al., 2005). In addition, it was reported that the effectiveness of serum in vitro embryo production (IVP) might change considerably from one batch to another. Because of these reasons, there has been a trend to use more defined proteins, such as bovine serum albumin (BSA) instead of undefined natural serum preparations like fetal calf serum (FCS) and estrus cow serum (ECS) (Sagirkaya et al., 2007). However, BSA has been shown to be ill-defined due to its affinity for biological contaminants and the variability in quality of commercial preparations. Furthermore, the risk of spreading infectious diseases via laboratory produced embryos resulting from contaminated protein supplementation in culture media is well recognized (Sirisathien et al., 2003).

Blood serum seems to be one of the ingredients of culture media that provides nutrients as well as physical components such as pH and or chelating actions. Serum or BSA are also sources of colloidal particles, which can be as effective, osmotically, as a single molecule or ion; the osmotic pressure of blood colloids is only 0.5–1% of the total osmotic pressure of blood, yet the blood colloids become the determining factor in the flow of

fluids across physiological membranes. Another physical component provided by both serum and BSA are surface-active properties (Palasz et al., 2000).

Various sera are used as media supplements of oocytes culture. The maturation media and the selection of protein supplements and hormones for IVM play an important role in subsequent IVF and in vitro development (Peluso and Hirschel, 1987). The addition of sera to culture media has been shown to improve the in vitro survival rate of mouse oocytes and prevent hardening of the zona pellucidu (Cross and Brinster, 1970; Eppip and Schroeder, 1986). In more IVM studies, serum was used as source of protein and growth factor (Hewitt et al., 1998; Mahi et al., 1993; Chian, 2004).

Bovine serum in the form of FCS or ECS, fetal bovine serum (FBS) and BSA have been employed not only in bovine but also in sheep oocyte in vitro maturation studies (Agrawal and polge, 1988; Braun, 1988; Crosby et al., 1971). Although several observations have been shown using umbilical cord serum (UCS) as a replacement of bovine serum in the maturation medium (Lee et al., 1998; Zhang et al., 2007).

Recently, human umbilical cord serum (HUCS) used in the IVM and culture of human and mouse oocytes (Lee et al., 1998; Zhang et al 2007). Cord blood serum is a rich source of cytokines required for growth and survival of different types of stem cells. The placental trophoblast cell produces a variety of growth factor like: insulin-like growth factor (IGF), Transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF). Broxmeyer et al, (1994) have assayed for a number of such co-stimulating factors present in cord blood plasma (Broxmeyer et al., 1994).

Lee et al, (1998) reported the percentage of gonadotrophins (FSH, LH) and steroids (estrogen, progesterone and testosterone) in the HUCS. Also showed that using of HUCS in IVM increase maturation rate and cumulus expansion of cumulus oocytes complexes (COCs) (Lee et al., 1998). Zhang et al (2007) showed that supplementation of HUCS in maturation medium increases maturation rate and high quality embryos (Zhang et al., 2007).

UCS are very precious serving as a source of stem cells and are usually collected for transplantation to cure patients with e.g., leukaemia (Zhang et al., 2007). There is not any information about UCS as a supplement to IVM medium for sheep oocyte maturation. The objective of the present study was to determine if HUCS, ovine umbilical cord blood serum (OUCS) and bovine umbilical cord blood serum (BUCS) added to IVM medium would improve oocyte maturation and subsequent early embryonic development of sheep oocytes.

Chapter 2

Literature Review

2.1. In vitro embryo production (IVEP)

In vitro embryo production (IVEP) in small ruminants provides an excellent source of low-cost embryos for basic research on developmental biology and physiology, and for the commercial application of the emerging biotechnologies such as nuclear transfer and transgenesis (Baldassare et al., 2002). The successive steps of IVEP of embryos are: in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) (Li et al., 2006). Despite the achievement of oocyte maturation and culture in vitro, oocyte competency and embryo developmental rate are still reduced compared to oocytes produced in vivo. Moreover, in vitro-produced embryos display many differences compared to their in vivo-derived counterparts (Sirard et al., 2007).

2.2. History

The 21st century seems set to see a revolution in the application of biotechnological procedures to farm animals. This revolution has been heralded by the production of transgenic mice by Brinster et al (1982), followed rapidly by the production of transgenic cattle, sheep and pigs, the development of practicable sperm sexing in cattle and the dramatic production of the sheep, Dolly, cloned from a somatic cell by Campbell et al (1996) and Wilmut et al (1997) with all the possibilities that offers for the production of genetically modified animals (Kane, 2003).

2.2.1. Milestones in IVM and IVF

Investigators of mammalian oogenesis usually refer to oocyte maturation as the set of processes occurring from the germinal vesicle stage to completion of the second meiotic division with the formation of the first polar body. Milestones in the study of in vitro maturation of oocytes include the discovery by Pincus and Enzmann in 1935 that release of immature rabbit oocytes from the inhibitory influence of the follicle allowed them to resume maturation in culture, and the demonstration by Minato and Toyoda in Japan in 1983 and by Schroeder and Eppig in 1984 in the USA that in vitro matured mouse oocytes

fertilized in vitro could result in live offspring. Other milestones include the finding that 39 °C was the optimal temperature for maturation of cattle oocytes, the birth of live calves from in vitro matured and fertilized cattle oocytes and the birth of a live mouse pup after complete development from primordial oocyte to in vitro fertilized embryo and transfer to a host mother. This last work, which was carried out in Eppig's laboratory, involved a two step procedure for maturation of the oocytes in which the ovaries of new born mice were first cultured for a number of days, the ovaries then dissociated with enzyme, the oocytes–granulosa cell complexes matured in vitro, fertilized and cultured in vitro to the 2-cell and blastocyst stages. The live pup was born after transfer of a 2-cell embryo to the oviduct (Kane, 2003).

Milestones in IVF include the discovery of the need for sperm capacitation in rabbits by Austin (1951) and Chang (1951), the definitive proof of in vitro fertilization in rabbits by Chang (1959), the birth of the first human in vitro fertilized baby in 1978 as a result of the work of Edwards, Steptoe and co-workers, the birth of the first in vitro fertilized calf in Brackett's laboratory, the discovery that 39 °C was the optimal temperature for bovine IVF and the development of improved methods for in vitro capacitation of bull sperm such as the use of heparin (Kane, 2003).

2.2.2 Milestones in embryo culture

Milestones in the development of in vitro culture of embryos include the first attempt at mammalian embryo culture by Brachet (1912) using coagulated blood plasma to culture 5–7 day rabbit blastocysts in glass dishes, the cinematographic studies of Lewis and Gregory (1929) on cleaving rabbit embryos, and the discovery by Hammondin 1949 that 8-cell mouse embryos could be cultured to blastocysts in a simple salt solution supplemented with hen egg white and yolk. This was followed by the absolutely crucial discovery of Whitten in 1957 that the addition of lactate as an energy source allowed culture of 2-cell mouse embryos to blastocysts in a simple Krebs Ringer bicarbonate solution supplemented with serum albumin, and the demonstration by McLaren and Biggers (1958) that embryos cultured to the blastocyst stage in this simple medium developed to live young after transfer to host mothers. This discovery of Whitten's, which was subsequently extended by the work of Brinster, Biggers, Whitten and their collaborators, laid the basis for the subsequent huge expansion of research into preimplantation development in the mouse and other species and without this discovery embryo biotechnology would not exist. Later

milestones in embryo culture include the finding in Foote's laboratory that, unlike the mouse, amino acids were essential for blastocyst formation in the rabbit, and the cleavage and development to blastocysts of in vitro fertilized human embryos. A major milestone for farm animal embryo culture was the discovery in First's laboratory in 1983 of the importance of using a temperature equal to the animal's core body temperature (about 39 °C for cattle) for in vitro maturation and fertilization of cattle oocytes; this led to the later realization that the same temperature should be used for sheep and cattle embryo culture. A further very significant factor in the development of embryo culture in farm animals was the finding that embryos tended to develop better in gas phases with oxygen concentrations lower than 20%, especially when cultured in the absence of somatic cell support (Kane, 2003).

2.3. In vitro maturation

In vitro maturation is probably the most critical part of the whole process of in vitro embryo production. Ruminant oocytes are usually matured at 39°C under a 5% CO_2 in a humidified atmosphere. The optimal maturation time (more than 90% of the oocytes at the metaphase II stage) are 22-24h. Due to the high lipid content of oocytes cytoplasm, it is not possible to follow the progression of their nuclear status during culture. Additionally, the presence of the cumulus cells is required during IVM for efficient cytoplasmic maturation and these cells mask the oocyte. Consequently, the only visible sign of oocyte maturation during IVM is the expansion of the cumulus cells. These cells produce hyaluronic acid which is secreted and polymerized in the extracellular matrix, leading to the increase of intercellular space.

Several culture media have been proposed for IVM (MEM, Waymouth, and Ham-F12). However, the most widely used seems to be the tissue culture media-199 (TCM199) medium, bicarbonate buffered and containing minerals, carbon and energy sources (glucose, glutamine) as well as vitamins and amino acids. The medium is generally supplemented with high molecular weight molecules that exert a surfactant effect (bovine serum albumin; BSA) and provide hormones and growth factors (fetal calf serum (FCS), serum of female in estrus, follicular fluid). These complex additives from animal sources may raise sanitary questions due to possible presence of pathogen agents, and decrease the reproducibility of experiments. They may be successfully replaced by high molecular weight polymers (Ali and Sirard, 2002) for the surfactant effect (such as polyvinyl alcohol)

and by cocktails of purified or recombinant hormones and growth factors (Lonergan et al., 1996).

Hormones are usually added to the maturation medium (FSH, LH, estradiol), in addition to biological fluids. Their exact function is not clearly established, althrough the effect of FSH on cumulus expansion and metabolism has been clearly established (Sutton-McDowall et al., 2004). Estradiol effect on oocyte is exerted through intracellular receptors and seems to be detrimental to meiotic progression, inducing chromosomal abnormalities (Beker-van Woudenberg et al., 2004). Growth hormone (GH) stimulates some aspects of cytoplasmic maturation (cortical granules migration) as well as nuclear maturation, resulting in a higher blastocyst yield (Bevers and Izadyar, 2002). GH action seems to be mediated by cumulus cells (Izadyar et al., 1997). Epidermal growth factor (EGF) has been shown to stimulate nuclear and cytoplasmic maturation in a wide variety of species including mouse, pig, cattle, deer goat and sheep (Abeydeera et al., 2000; Cognie et al., 2004). High blastocysts yields could be obtained after maturation of cattle oocytes in TCM199 only supplemented by EGF. EGF action is exerted, at least partly, directly on the oocyte and recent studies showed that EGF family of growth factors (epinephrin, amphiregullin, betacellulin) could be produced by granulosa cells under gonadotropins stimulation and could thus mediate LH signaling inside the follicle, transmitting the ovulatory signal to the oocyte (Park et al., 2004). Insulin like growth factor-I (IGF-I) has also a positive effect on cytoplasmic maturation of cattle oocytes (Bevers and Izadyar, 2002). Indeed, the regulation of IGF-I and II through the controlled expression and degradation of IGF binding proteins could be one of the major processes of control of late follicular growth (Mazerbourg et al., 2003).

Another important aspect of COC environment during IVM is the antioxydative potential of the IVM medium (Ali et al., 2003). As all living cells, oocyte is sensitive to reactive oxygen species (ROS) that could induce detrimental chemical reactions (lipid or protein peroxydation). To overcome possible ROS detrimental effects during culture, antioxydative molecules (β-mercaptoethanol, cystein, vitamin A, C, E), divalent cations chelators (EDTA, taurine, hypotaurine, transferrine) or ROS scavengers (superoxyde dismutase, catalase) could be added to maturation medium (Blondin et al., 1997). However, COC are capable to produce their own protection system by secreting ROS scavenger enzymes like superoxyde dismutase and catalase (Cetica et al., 2001) or through the production of intracellular reducers molecules like glutathion (de Matos and Furnus, 2000). Stimulating these endogeneous protection systems may help oocyte to survive

challenging in vitro conditions. The addition into IVM medium of cysteamine, a cell permeable precursor of cysteine, itself involved in glutathion (GSH) synthesis, has been shown to increase GSH concentration in oocytes and, in turn, to increase the resulting developmental competence after IVM in different species (Cognie et al., 2004). Surrounding cumulus cells are also contributing to establishment of high GSH content in the oocyte (Luciano et al., 2005). Therefore, stimulating cumulus cells may also help to protect oocytes against environment ROS.

It has been established for a long time that the presence of cumulus cells is required for the success of oocyte cytoplasmic maturation and fertilization (Gilchrist et al., 2004). Therefore, maintaining the viability and functionality of cumulus cells is likely to be another important challenge for IVM success. Oocyte secreted factors (OSF) are known to drive cumulus cell differentiation during follicular growth (Li et al., 2000) and to allow cumulus expansion during oocyte maturation (Dragovic et al., 2005). The precise identification of the active factors is not fully established. However, GDF-9 and BMP-15, two members of the transforming growth factor-family (TGF-family) of growth factors, are specifically produced by the oocyte and their effect on cumulus cells, and in turn on matured oocyte developmental competence, have been recently established (Hussein et al., 2006). Since these factors are able to protect cumulus cells against apoptosis that appear in culture (Hussein et al., 2005), their addition to IVM medium may be recommended, at least to help at maintaining cumulus functionality in culture.

2.3.1. Nuclear maturation

In the ovary, all primary oocytes are blocked at the prophase stage of the meiotic cycle the block of meiotic progression during follicular growth is controlled by unknown factors produced by the surrounding somatic cells (Mermillod and Marchal, 1999). At this stage, the round shaped nucleus, called the germinal vesicle (GV), contains diffuse chromatin. During early folliculogenesis, the oocyte grows; undergo strong morphological modifications and stores molecules (proteins, RNAs) that will be determinant for the success of fertilization and early embryo development. The zona pellucida, a glycoprotein envelope surrounding the oocyte membrane, is synthesized by the oocyte during this period of growth. The zona pellucida has important function in regulating fertilization and protection of the embryo until hatching. The oocytes resume meiosis and progress to the metaphase II stage only if they encounter a gonadotropin surge in the preovulatory follicle.

They stop their progression at this MII stage until fertilization in the oviduct, after ovulation.

Oocytes that are collected from growing follicles for IVP are blocked at the prophase stage of meiosis. As soon as they are removed from the follicular inhibitory environment; meiotic resumption occurs spontaneously and progress to metaphase II (Sirard, 2001). This spontaneous meiotic resumption of the oocyte outside of the follicle is the basis of in vitro maturation.

2.3.2. Cytoplasmic maturation

Cytoplasmic maturation covers all morphologic and molecular events accompanying nuclear maturation after LH surge in preovulatory follicles and preparing oocyte cytoplasm to successful fertilization and embryo development. Cytoplasmic maturation includes well known morphological modifications, such as the migration of cortical granules in the cortical region of the ooplasm (Cran, 1989). These granules are stored during oocyte growth and release their enzymatic content in the perivitelline space after fertilization. These enzymes modify the structure of the zona pellucida, preventing the penetration of additional spermatozoa. Meiotic competence is acquired during early folliculogenesis, soon after the apparition of the antral cavity in the follicle (Mermillod et al., 1999). However, after this acquisition, the oocyte requires a further differentiation period during late follicular growth to reach the full competence for cytoplasmic maturation (Sirard et al., 2006). This late differentiation occurs under the meiosis inhibiting signal sent by somatic follicular cells that maintain the meiotically competent oocyte at the prophase stage.

During IVP, more than 90% of the oocytes collected from follicles larger than 3 mm in cattle are able to complete nuclear maturation. However, few of them are competent for cytoplasmic maturation. Consequently, only 30 to 40% of the oocytes reach the blastocyst stage after IVF and IVD (Figure 2.1). This contrast highlights the functional importance of cytoplasmic competence (Sirard et al., 2006). When oocytes harvested from larger follicles or in vivo matured oocytes are processed under the same IVF – IVD techniques, the success rate is increased (Hue et al., 1997; Lequarre et al., 2005; Marchal et al., 2002). Ovarian stimulation by gonadotropins increases the number of follicles growing to large size classes by protecting them against involution through the atresia process. Consequently, ovarian stimulation increases the number of competent oocytes collected through the ovum pick-up (OPU) process.