In The Name Of God

Razi University
Faculty of Agriculture
Department of Animal Science

M.Sc. Thesis

Title of the Thesis:

Assessment of developmental competence of bovine oocytes based on side of ovaries and presence or absence of corpus luteum

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February 2013

To

My father

My Mother,

And my sister

ACKNOWLEDGMENTS

I bow my head with great reverence to the almighty, who is omnipresent, omnipotent and omniscient.

The success of any venture depends upon the people who helped in its formation. I take this opportunity to thank the number of people who assisted me in this project.

I respectfully wish to express my deepest sense of gratitude and indebtedness to my major advisor Dr. H. Karami Shabankareh, for his judicious planning, critical appraisal, inspiring and sagacious guidance, inexorable supervision and support with absolute positive attitude without which, this study would have not been accomplished.

Sincere gratitude and indebtedness are due to Dr H.Hajarian for his great encouragement and remedial suggestions during the course of this study .

I would like to express deepest sense of gratitude to my coworker Engineer: Ms. Azimi for her friendly behavior and nice help in all the time.

I am grateful to Mr. Mehrdad Sadeghifor supplying the cow ovaries from local slaughterhouse.Thanks to Ms. Bahare Akhtarshenas and Mehrangiz Piri for helping in the IVF andET laboratory.

I wholeheartedly acknowledge the unrepayable contribution from my dear father , mother and affectionate sister.

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: February: 2013Mohammad Hamed Shahsavari

Abstract :

Bovine ovaries were collected from local abattoir and categorized based on side of ovaries (left or right) and presence or absence of corpus luteum (CL).This study was conducted in four separate experiments: In experiment 1, the developmental competence of bovine oocytes originating from left ovaries (L-oocytes), right ovaries (R-oocytes) and control ovaries (C-oocytes, without selection based on side of ovaries) was evaluated. After classification, the oocytes were maintained undisrupted in the in vitro production (IVP) process. In experiment 2, the brilliant cresyl blue (BCB) test was used as a selection criterion to evaluate the heterogeneity in the developmental competence of oocytes originating from left or right ovaries. After aspiration, cumulus oocyte complexes (COCs) were incubated in modified Dulbecco's phosphate-buffered saline (mDPBS) supplemented with 26 μM of BCB for 90 min and classified into two classes: oocytes with blue cytoplasm coloration (BCB^+-oocytes: more competent oocytes) and oocytes without blue cytoplasm coloration (BCB^--oocytes: less competent oocytes). Directly after classification, the oocytes were maintained undisrupted in the IVP process. In the control (C-oocytes) treatment: oocytes were cultured directly (without exposure to BCB) after recovery in the IVP process. In experiment 3 , the developmental competence of bovine oocytes originating from ovaries bearing a CL (CL^+-oocytes), ovaries not bearing a CL (CL^--oocytes) and control ovaries (C-oocytes, without selection based on presence or absence of a CL) was evaluated. In experiment 4 , the brilliant cresyl blue (BCB) test was used as a selection criterion to evaluate heterogeneity in the developmental competence of CL^+-oocytes or CL^--oocytes. In experiment 1, the percentage of cleavage and blastocyst rate of R-oocytes and C-oocytes were greater ($P < 0.001$) than that of L-oocytes. In experiment 2, results confirmed the observations in the first experiment in which the developmental competence of R-oocytes was greater than that of L-oocytes and it appears that there was homogeneity in the developmental competence of L-oocytes or R-oocytes. In experiment 3 , the percentage of cleavage and blastocyst rates of CL^--oocytes were greater than that of CL^+-oocytes. Similarly, in experiment 2, CL^+-oocytes had less percentage of BCB^+-oocytes (more competent oocytes) than CL^--oocytes. In conclusion, current results confirmed that side of ovaries and presence or absence of corpus luteum affected the developmental competence of all oocytes and oocytes originating from right ovaries and ovaries not bearing a CL had greater developmental competence than that of oocytes originating from left ovaries and ovaries bearing a CL, respectively.

Key words: in vitro production, brilliant cresyl blue, cumulus oocyte complexes, cleavage, blastocyst.

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Chapter 1

Introduction

In embryonic development there should be equal activity of both sides of the reproductive system (Gereš et al., 2011). Two ovaries usually take an equal part in the process of follicle ripening and ovulation and there is no definite preference of one ovary over the other during consecutive cycles (Bouchlariotou et al., ,2012). However there is much evidence of the influence of functional asymmetry on fertility parameters in older literature. The right ovary in humans produces more ovulations (Fukuda et al., 2000) and in IVF patients with healthy ovaries, the right ovarian responses are superior to the left ovarian responses (Lan et al., 2010). In rats, the right ovary is larger than the left (Mittwoch and Kirk, 1975) and in hamsters, mice and rats the right ovary contains more corpora lutea (CLs) (Fritzsche et al., 2000), while there is no difference in ovulation response between left and right ovaries in rabbits (Ju et al., 2001). In farm species, Arthur (1958) described ovarian activity in the mare and noted a greater proportion of corpora lutea present on the left ovary compared with the right ovary.

Follicular oocytes recovered from ovaries of slaughtered cattle are commonly used to study the processes of maturation, fertilization and the technique of in vitro embryo production (IVEP). There are distinct problems associated with IVEP of bovine embryos. Several studies noted that the intrinsic quality of the oocyte is the key factor in determining the proportion of oocytes developing to the blastocyst stage (Lonergan et al., 2003; Alm et al., 2005). These problems include or are derived from the fact that the origin of the oocytes used (stage of estrous cycle, stage of follicular wave, origin of ovaries, etc.) is unknown and therefore oocyte quality is very variable (Lonergan and Fair, 2006; Karami-Shabankareh and Mirshamsi,2012). It was demonstrated that ovulation switching from the left to the right ovary in two successive cycles may increase the chances of pregnancy during intrauterine insemination and/ or IVF natural cycles (Fukuda et al.,2006).

The corpus luteum (CL) is a transient endocrine gland that forms in the ovary of mammals and some non-mammals from the follicular layers after ovulation. The evolution of the CL is associated with viviparity in mammals and is necessary for the production of progesterone throughout the luteal phase of the estrous cycle to maintain pregnancy (if a conceptus is present) and during pregnancy, to decrease gonadotrophin secretion and

prevent behavioral estrous occurring (Karami Shabankareh et al , 2009 ; Tomac et al.,2011). Information on the structure of CL (including ultrastructure), histochemistry, biochemistry, endocrinology, immunology, *in vitro* manipulation by growth factors and regulatory mechanisms (including apoptosis), has been elaborated especially in rodents and large ruminants (Karami Shabankareh et al 2010; Shabankareh et al.,2013).

Previous studies confirmed asymmetry in the function of the reproductive organs in dairy cows, due to differences in ovarian activity and probably even more because of physiological differences in the tubular part of the reproductive organs, depending on the side of previous gestation and involution of the postpartal horn of the uterus (Gereš et al., 2011). An important finding in this case was the existence of not only a systemic, but also a possible intraovarian effect of the CL on the follicular population (Bartlewski et al., 2001; Karami Shabankareh et al., 2009). There was a relationship between the development of CL and the development of follicles (Contreras-Solis et al 2008). Karami Shabankareh et al. (2010) have suggested that the serum oestradiol concentration and follicular population are mainly related to the presence or absence of a corpus luteum. According to Bellin et al. (1984), follicles developed on ovaries containing CL from a previous gestation have a smaller diameter than those on ovaries without CL from a previous gestation. Matton et al. (1981) considered that CL directly inhibits folliculogenesis, because there is a larger number of developing middle size follicles during the whole oestrous cycle on ovaries without CL from a previous gestation compared to ovaries containing one. The presence of the CL was shown to locally suppress the numbers of antral follicles not growing beyond 3 mm in diameter in Western White Face ewes (Bartlewski et al., 2001). However, in another study, it has been surmised in ewes, that the CL acts locally to increase the numbers of all follicles visible on the ovarian surface (Dailey et al. 1982). These observations indicate an absence of dominance effects during the active phase of the CL, giving new evidence to support the hypothesis of Adams (1999) about the existence of suppressive effects of progesterone from the CL on lifespan of dominant follicles. Islam et al. (2007) reported that higher number of follicles aspirated and normal cumulus oocyte complexes (COCs) were found in CL-absent group compared to those of CL-present group of ovaries. On the other hand, various biochemical constituents of follicular fluid from follicles in ovaries without a CL and ovaries with a CL, might indicate the existence of a possible local effect of the corpus luteum (Karami Shabankareh et al., 2010; Shabankareh et al., 2013).

Selecting competent oocytes likely to develop, is crucial for in vitro embryo production. Morphological evaluation alone is insufficient to distinguish competent oocytes that have the ability to bring about full-term pregnancy (Alm et al., 2005). With the urgent need for establishing non-invasive option for embryo selection, the brilliant cresyl blue (BCB) test has been successfully used to differentiate oocytes (Bhojwani et al., 2007; Mirshamsi and Karami Shabankareh,2012; Karami-Shabankareh and Mirshamsi, 2012). The BCB test is then based on the capability of G6PDH to convert the BCB stain from blue to colorless (active G6PDH: BCB^{-}, inactive G6PDH:BCB^{+}) (Rodriguez-Gonzalez et al., 2002). Several studies noted that the percentage of BCB^{+} oocytes developing to the morula and blastocyst stage were significantly higher than the control and BCB^{-}oocytes (Karami-Shabankareh and Mirshamsi, 2012 ;Mirshamsi et al., 2013). However, in other studies, the BCB selection had no beneficial effects on the development of oocytes (Alm et al., 2005) or impaired the developmental ability of oocytes to the blastocyst stage (Wongsrikeao et al., 2006).

On the basis of our knowledge, this is the first investigation attempting to evaluate the developmental potential of oocytes derived from left (L-oocytes) and right (R-oocytes) ovaries and ovaries bearing a CL (CL^{+}-oocytes) or ovaries not bearing a CL (CL^{-}-oocytes) following IVEP. Consequently, the initial objective of the study was to compare the developmental competence of L-oocytes, R-oocytes and CL^{+}-oocytes, CL^{-}-oocytes. The second objective was to evaluate heterogeneity in the developmental competence of oocytes originating from left or right ovaries and ovaries bearing a CL or ovaries not bearing a CL using the BCB test as a selection criterion.

Chapter 2

Literature Review

2.1. *In vitro* embryo production (IVEP)

The number of oocytes that reach ovulation is obviously limited to the number of offspring that each species canbring to term in the uterus. From the several thousand primary oocytes available at birth, the number of oocytes that will be fertilized and develop to term following naturalmating or artificial insemination is reduced to only a few. Reproductive technologies initially overcame this limit by collecting pre-implantation embryos from valuable donor animals that were super ovulated and transferring them to recipient animals. It was soon evident that collecting oocytes instead of embryos would increase the efficiency ofthis approach. Moreover, the further away from the time of ovulation the collection takes place, the greater the number of available oocytes (Merton et al 2003). In theory, the earlier the collection takes place (i.e. at birth, or even before birth,when the pool of several thousand primordial follicles canbe exploited), the greater the number of oocytes with the potential to be fertilized and developed to term(Gandolfi et al.,2005).

In vitro embryo production (IVEP) in 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). *In vitro* embryo production is a newer and more flexible approach, although it is technically more demanding and requires specific laboratory expertise and equipment that are most important for the quality of the embryos produced (Walker et al., 1992; Smeaton et al., 2003; Lucci et al., 2004; Tominaga and Hamada, 2004) .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 . 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 oocyte–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(Eppig and O'Brien, 1996).

A vast number of historical aspects of IVF have been reviewed in detail by Wright and Bondioli (1980) and Biggers (1984). The first attempt to fertilize mammalian ova *in vitro* was made by Schenk (1878) using rabbits and guinea pigs. Later, Walter Heape (1890, 1898) showed that fertilized rabbit ova could be successfully flushed and transferred to a surrogate mother. There was then an interval until Pincus and Enzmann (1934) published results of studies on IVF of rabbit. In humans, several attempts and claims of success to fertilize ova *in vitro* were reported by Rock and Menkin (1946) and Menkin and Rock (1948), but the results were unconvincing to many investigators. Finally, the first baby was

born to a woman without fallopian tubes following IVF and embryo transfer (Steptoe and Edwards 1978). In domestic farm animals, early work using ovulated oocytes resulted in sperm penetration and cleavage following IVF (Bondioli and Wright 1980,Thampson et al 1989). Quirke and Gordon (1971) and Crosby et al (1971) observed some evidence of fertilization when *in vitro* matured sheep follicular oocytes were inseminated in vivo. Dahlhausen et al (1980) attempted IVF of *in vitro* matured follicular oocytes which resulted in 4 out of 6 oocytes showing an extruded second polar body. In cattle, results of early IVF experiments using IVM procedures with a variety of media and conditions were not encouraging (Baker and Polge 1976, Bregulla et al 1974) only recently in the 1980's was the first calf born as a result of IVF, as reported by Brackett et al (1982 ,1984), followed by goats (Hanada 1985a) sheep (Hanada 1985 b), and pigs (Cheng et al 1986).

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 Hammond in 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 . 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 $^{\circ}$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). The early steps in development including maturation, fertilization, and timing of first cleavage, activation of the embryonic genome, compaction, and blastocyst formation can be affected by the culture media and conditions as well as the production procedure itself (Wrenzycki et al., 2007).

2.3 *In vitro* maturation (IVM)

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