In The Name Of

God

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Razi University

Faculty of Agriculture
Department of Animal Science

M.Sc.Thesis

Title of the Thesis

Comparison of the effect of different sera and existent hormones in related sera on in vitro maturation and early embryonic development of sheep oocytes

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By:
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2009

Dedicated to:

My father,

My mother

My husband

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

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Dated: October. 2009 *Vida. K. jasemi*

Abstract

In the present study, the main objective was to investigate and compare the effect of different sera with existent hormones in related sera on the cleavage and developmental capacity of ovine oocytes. Thus, two experiments were designed.

In Exp.1, In separate experiments, the basic culture medium was supplemented with 10 % human pregnant serum (HPS), 10 % human menopausal serum (HMS), 10 % pregnant mare serum (PMS) and 10% estrous mare serum (EMS). The basic culture medium (control) was comprised of the TCM-199 supplemented with 0.5 μg/mL LH, 0.5 μg/mL FSH, 1 μg/ml 17β estradiol, 0.23 mM Na pyruvate, 50 μg/ml gentamycin sulphate and 0.01% PVA.

Cleavage rates of embryos derived from IVM with HPS, HMS, PMS and EMS were reduced compared with controls (61.3, 60.1, 62.8 and 62.2 % and 87%; respectively; $P < 0.05$). Higher morula rates were obtained in oocytes cultured with HMS and EMS (57.4 and 59.8 %, respectively; $P < 0.05$). The incidences of blastocyst formation were higher in the HMS and EMS groups (43.3 and 43%, respectively; $P < 0.05$) than in the control group (26.8%).

In Exp.2, The basic culture medium was supplemented with various concentrations of existent hormones in related sera. The in vitro maturation was carried out in five different treatment groups (I) basic culture medium only , (II) basic culture medium + human pregnant serum hormones (HPSHs,10.2 mlu/ml FSH, 34.8 mlu/ml LH , 353.3 pg/ml estrogen, 142.4 ng/ml progesterone, 1991 IU/L βhCG), (III) basic culture medium + human menopausal serum hormones (HMSHs, 81.8 mlu/ml FSH , 54.5 mlu/ml LH, 8.5 pg/ml estrogen, 0.1 ng/ml progesterone), (IV) basic culture medium + pregnant mare serum hormones (PMSHs, 69.8 mlu/ml FSH, 37.2 mlu/ml LH, 297 pg/ml estrogen, 6.5 ng/ml progesterone, 98 ng/ml eCG) and (V) estrous mare serum hormones (EMSHs, 45.7 mlu/ml FSH, 68.5 mlu/ml LH, 53.2 pg/ml estrogen, 0.2 ng/ml progesterone).

cleavage rates of embryos derived from IVM with HPSHs, HMSHs, PMSHs and EMSHs were reduced compared with controls ($P < 0.05$). Higher morula and blastocyst rates were obtained in oocytes cultured with HMSHs and EMSHs ($P < 0.05$).

Results of T-test showed that, There were significant differences in rates of cleavage, morula and blastocyst between HPS VS HPSHs and PMS VS PMSHs. There was no significant difference in cleavage and morula rates between EMS and EMSHs but there was significant difference in rate of blastocyst between EMS and EMSHs($P < 0.05$). There was no significant difference in morula rate between HMS and HMSHs ($P < 0.05$).

It is concluded that ingredients of HPS, HMS, PMS and EMS such as FSH, LH, eCG, βhCG, estrogen and progesterone exhibit wide variations. These variations may cause different results in IVM, IVF and subsequent embryo development.

Key words: oocye, maturation, serum, ovine

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Chapter 1
Introduction

Ovine in vitro embryo production is one of the future sheep breeding strategies for the development of biotechnology of in vitro embryo production (IVEP). The successive steps of IVEP are: in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC). Successful maturation of oocytes is a prerequisite for fertilization and further embryo development. Several workers have studied different aspects of IVM in mammalian oocytes. In most IVM studies, the basic medium is supplemented with serum or crystallized albumin. The fetal calf serum has been widely used for oocyte maturation in vitro in many species such as goat, bovine , pig, water buffalo, horse and llama. Furthermore the estrous goat serum (EGS), estrous sheep serum (ESS) and estrous mare serum (EMS) have been used for oocyte maturation in vitro in many species.

To the best of our knowledge, however, there was not much information about the relationship between existent hormones in human pregnant serum (HPS), human menopausal serum (HMS), pregnant mare serum (PMS) and estrous mare serum (EMS) and efficiency of these sera on in vitro maturation of sheep oocytes. Human chorionic gonadotrophin (hCG) has been detected in both blood and the urine of pregnant women as early as 8 d after conception by sensitive radio immunoassays. hCG has both LH- and FSH-like actions, but predominately LH-like biologic actions. Lin et al (2008) reported that; there was a trend of decreasing maturation and fertilization rates with increasing concentrations of serum from pregnant women. In menopause women, ovaries naturally fail to produce estrogen and progesterone when they have few remaining egg cells. At that stage, the ovaries become less able to respond to the pituitary hormones: follicle stimulating hormone (FSH) and luteinizing hormone (LH) and less estrogen is produced. Levels of FSH and LH subsequently rise and a measurement of FSH is sometimes used to diagnose menopause. It has been shown that human serum was more effective for one cell sheep embryo culture, than sheep serum itself.

During equine gestation, cells from the chorionic girdle of the equine trophoblast produce equine chorionic gonadotrophin (eCG) that can be found in circulation from day 36 until about day 130 of gestation . eCG has both FSH and LH biologic actions, with the FSH actions being dominant. Recently it has been shown that a short exposure of oocytes (60–240 min) to eCG promoted resumption of meiosis and progression of canine oocytes to MI stage.

The mare's estrous cycle is defined as the period from one ovulation to the subsequent ovulation, with each ovulation being accompanied by signs of estrus and plasma progesterone concentrations below 1 ng/mL. Figueiro et al (2002) reported that, the EMS can be used for the IVP of bovine embryos. The estrous cycle consists of two phases, the follicular phase (estrus) and the luteal phase (diestrus). The duration of the mare's estrous cycle normally ranges from 19.1 to 23.7 days , consisting of 14 days of diestrus and 7 days of estrus, when the mare is sexually receptive. The cycle is also under a control of factors from the ovaries. The ovaries produce hormones, estradiol and progesterone, which through negative or positive feedback control the release of FSH and LH. The estrous phase will typically last from five to seven days. The increasing amounts of estrogen from the dominate follicle signals the brain to increase production of LH. During this period, FSH concentrations are normally decreased while LH is rising to a peak 1 or 2 d after

ovulation.

It is fair to assume that FSH, LH, estrogen, progesterone, eCG and βhCG have effects on oocyte competence for IVM and fertilization. Therefore, the objectives of this project were to: (1) test the relationship between in vitro maturation of ovine oocytes and measures of women and mares serum gonadotrophin (FSH, LH, eCG and βhCG) and steroids (estrogen and progesterone) hormone levels, and (2) comparison of the effects of sera and existent hormones in related sera on in vitro maturation and early embryonic development of sheep oocytes.

Chapter 2
Literature Review

2.1. In vitro embryo production (IVEP)

The technology of in vitro embryo production (IVEP) is taking on more importance in view of its applications in the emerging biotechnologies like cloning and transgenic animal production.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). In this reason we focus on IVM that has a critical effect on oocyte competency and embryo developmental.

2.1.1. In vitro maturation

Oocyte maturation involves cytoplasmic as well as nuclear maturation. Cytoplasmic maturation entails the accumulation of factors in the ooplasm that are required for resumption of meiosis, fertilization and embryonic development. Nuclear maturation includes the events that occur associated with the resumption of meiosis, which results in a haploid oocyte and preparation of the oocyte for fertilization (Wasserman and Albertini, 1994). The physiological signal that triggers resumption of meiosis in prophase I arrested oocytes is not well understood. Following follicular recruitment and oocyte growth, the oocyte is capable of undergoing resumption of meiosis and fertilization. Resumption of meiosis in explanted follicles cultured in vitro is induced by gonadotrophins (Tsafriri and Dekel, 1994). In large Graafian follicles, FSH, LH and hCG all triggered resumption of meiosis (Hillensjo 1976; Tsafriri et al., 1976). In rats, oocytes in small antral follicles cultured in vitro resumed meiosis in the presence of FSH and not LH (Dekel et al., 1988). This observation is consistent with the fact that no LH receptors are found in small antral follicles in rodents (Eppig et al., 1997).

2.1.1.1. Nuclear Maturation

COC extracted from follicles are immature and arrested in prophase I of meiosis. Immature oocytes are characterized by the presence of a nuclear envelope that contains decondensed chromosomes (Wasserman and Albertini, 1994). Chiasmata and meiotic recombination has occurred before oocytes become arrested at this stage. Oocytes displaying a GV are not able to undergo fertilization or zona pellucida reaction. Cumulus cells that surround the oocyte are compacted.

In vivo, oocytes undergo germinal vesicle breakdown (GVBD) or dissolution of the nuclear membrane in response to the gonadotrophin surge. In vitro, oocytes extracted from bovine follicles $\geq$ 2 mm in diameter or from antral follicles from mice undergo GVBD if cultured in either the presence or absence of gonadotrophins. Oocytes in this stage display chromosome condensation and the initiation of cumulus cell expansion. Remnants of the nuclear envelope may still be visible. Spindle assembly begins and one kinetochore forms per chromosome rather than one per chromatid (Wasserman and Albertini, 1994).

Following GVBD, condensed chromosomes line up forming the metaphase I (MI) plate. The paired chromosomes begin moving to opposite ends (poles) of the cell during

anaphase I(Wasserman and Albertini , 1994).

Metaphase II (MII) stage oocytes are characterized by the presence of the first polar body and the formation of a nuclear envelope. The oocyte is now haploid but each chromosome has two chromatids. The oocyte is ready to undergo fertilization and support post-fertilization embryonic development(Wasserman and Albertini, 1994).

2.1.1.2. Cytoplasmic Maturation

Cytoplasmic maturation involves a variety of metabolic, biochemical and structural modifications required for the progression of meiosis, fertilization and the activation of pathways required for embryonic development (Izadyar et al., 1998). Structural modifications include the redistribution of organelles such as cortical granules, mitochondria, Golgi apparatus and endoplasmic reticulum. Biochemical and metabolic modifications include the acquisition of the ability to decondense sperm chromatin (Izadyar et al., 1998, Ducibella et al., 1993) and acquisition of stored mRNA (Gosden, 2002).

2.1.2. In vitro fertilization

Fertilization occurs only when a spermatozoon penetrates the oocyte. In order for fertilization to occur, the spermatozoa must first undergo capacitation in the female reproductive tract, in which the protective plasma coating covering the spermatozoa surface molecules is removed to allow sperm to be able to bind to the oocyte. Then spermatozoa adhere to the oocyte's zona pellucida to induce the acrosome reaction, in which the fusion of the spermatozoal plasma membrane and the outer acrosomal membrane occurs to allow the acrosomal enzymes to be released. Only after these two critical events are completed can fusion of the two gametes occur. Surrounding the oocyte are approximately 3,000 cumulus cells (Lin et al., 1994) embedded in a thick extracellular matrix which presents an unavoidable obstacle for spermatozoa. One of the significant components comprising the extracellular matrix is a specific disaccharide, hyaluronic acid. Spermatozoa posses an enzyme called hyaluronidase which breaks down hyaluronic acid found throughout the extracellular matrix surrounding the oocyte, thereby creating a pathway so that spermatozoa can travel towards and adhere to the zona pellucida. Drobnis and Katz (1991) observed using videomicroscopy spermatozoa that had undergone the acrosomal reaction on the zona pellucida were able to work through their acrosomal shroud and penetrate into the oocyte and those recovered from female reproductive tracts prior to reaching the oocyte had not undergone the acrosome reaction (Suarez et al., 1983; Kopf and Gerton, 1991).

A glycoprotein, PH-20, found on the head of mammalian spermatozoon is homologous to that of hyaluronidase (Lathrop et al., 1990). Observations indicate PH-20 has hyaluronidase activity and spermatozoa without the PH-20 on their membrane can not traverse the cumulus cells (Lin et al., 1994). The activation of PH-20 occurs during spermatozoan transport and is regulated by deglycosylation (Deng et al., 1999). Seaton et al. (2000) observed that the PH-20 migrates during capacitation from the tail to the acrosomal domain and that PH-20 undergoes endoproteolytic cleavage to optimize hyaluronidase activity.

The IVF portion of the *in vitro* system is designed to closely resemble the steps that occur in vivo around the time of fertilization. The in vitro conditions are inferior to those found in vivo as evidenced by low rates of MPN formation and a high incidence of polyspermy found during fertilization (Hunter, 2000). Polyspermic zygotes have fewer inner cell mass numbers and abnormal cleavage patterns (Han et al., 1999). The IVF system consists of preparing the spermatozoa and the oocytes for syngamy and the

coincubation of oocytes and spermatozoa for a certain duration of time.

In vitro, spermatozoa must undergo capacitation prior to coincubation with the oocytes. This occurs by incubating the spermatozoa in media for 2 to 4 h prior to IVF (Nagai et al., 1984). The medium used is Tris-buffered medium (Berger and Horton, 1988) without antibiotics with the addition of 5 m*M* of caffeine and 5 m*M* of calcium ion which increases the rate of sperm penetration (Abeydeera and Day, 1997). The cumulus cells surrounding the oocytes are removed prior to IVF by mixing them with 0.1% hyaluronidase. This is done to allow the spermatozoa to reach the egg faster and more efficiently, and reduce the number of spermatozoa needed for IVF. The main focus of recent research is optimizing the IVF environment, spermatozoa number, and coincubation time. An increase in spermatozoa number correlated to an increase in penetration rate and incidence of polyspermy in the oocyte (Wang et al., 1991). After the spermatozoon has penetrated the oocyte, the cortical granules release their contents exocytotically into the perivitelline space surrounding the oocyte. The cortical granule contents alter the properties of the zona pellucida, creating a block against polyspermy (Dandekar and Talbot, 1992). The thickness of the zona pellucida and perivitelline space is significantly smaller in in vitro derived oocytes than in vivo derived (Wang et al., 1998) and may be a part of the reason behind the insufficient block of polyspermy of IVF oocytes. The insufficient modification of the zona pellucida and cortical granule may be due to the lack of a secreted oviducal factor in vitro (Kim et al., 1996; Kano et al., 1994).

2.1.3. In vitro culture

The final step of the in vitro production system is IVC. The IVC step begins after IVF, consisting of sustaining the sheep embryos in a suitable environment. This begins at the ootid stage of development and continues through the zygote stage and cleavage processes up to a desired point of termination. *In vitro* derived embryos at the two to four cell stages are able to be implanted into recipients and carried to term (Mattioli et al.,1989). The developmental competence of *in vitro* derived offspring is significantly lower than natural offspring with respect to growth rate and weight (Kazuhiro et al., 1999).

As in IVM and IVF, the *in vitro* system is not as successful in producing viable embryos as is the *in vivo* system (Prather and Day, 1998). *In vitro* derived embryos have lower and delayed cleavage rates and asynchronous pronucleus development compared to *in vivo* derived embryos (Laurincik et al., 1994). Further along in development, the number of nuclei of *in vitro* derived blastocyts is significantly lower than *in vivo* blastocysts (Machaty et al., 1999) and the failure to induce significant rates of pregnancy in live recipients using *in vitro* derived embryos is significantly lower than *in vivo* derived embryos (Rath et al., 1995). The optimum incubation parameters for IVC of porcine embryos is at 5% carbon dioxide and 5% oxygen (Berthelot and Terqui, 1996; Kikuchi et al., 2002). The greatest variable in optimizing the IVC of embryos is the medium used. All media used contains various salts in low concentrations (Beckmann and Day, 1993), energy sources (Kikuchi et al., 2002), and macromolecules (Petters and Wells, 1993). Similar to IVM, much experimentation has been done adding various supplements to the media.

2.2. Maturation of oocytes in vitro

The IVM portion of the *in vitro* system is designed to closely resemble that which occurs *in vivo*. The matemal contribution to the developrnent of the embryo is determined during formation and maturation of the female gamete, the oocyte. The ability of the oocyte to achieve the capacity to be fertilized is acquired early in oogenesis, Therefore, an understanding of oocyte development and physiology forms the basis of current oocyte selection and in vitro maturation techniques. Ovine follicles from 2 to 8 mm in diameter

are commonly used as a source of oocytes for embryo production. Oocytes can resume meiosis if removed from the follicle and cultured in vitro. The oocytes are arrested in the dictyate stage, a prolonged diplotene stage of the first meiotic division also known as the germinal vesicle stage. Primordial oocytes are surrounded by the primordial follicle, a single layer of squamous shaped granulosa cells. This describes the first phase of oocyte growth where growth of the oocyte and follicle are highly correlated. The second phase of growth is when the follicle continues to grow while the oocyte remains arrested until it reaches full adult size (115 nm) in the antral follicle (1.8 mm)(Figure 2.1). During the second growth phase, oocytes quadruple in size and depend on somatic cell (granulosa) – oocyte interactions via gap junctions. The granulose (cumulus) cells provide nucleotides, amino acids, phospholipids, substrates for energy utilization, and maintain the ionic balance of the oocyte. Cumulus cells also protect oocytes against oxidative stress-induced apoptosis (Tatemoto et al., 2000).

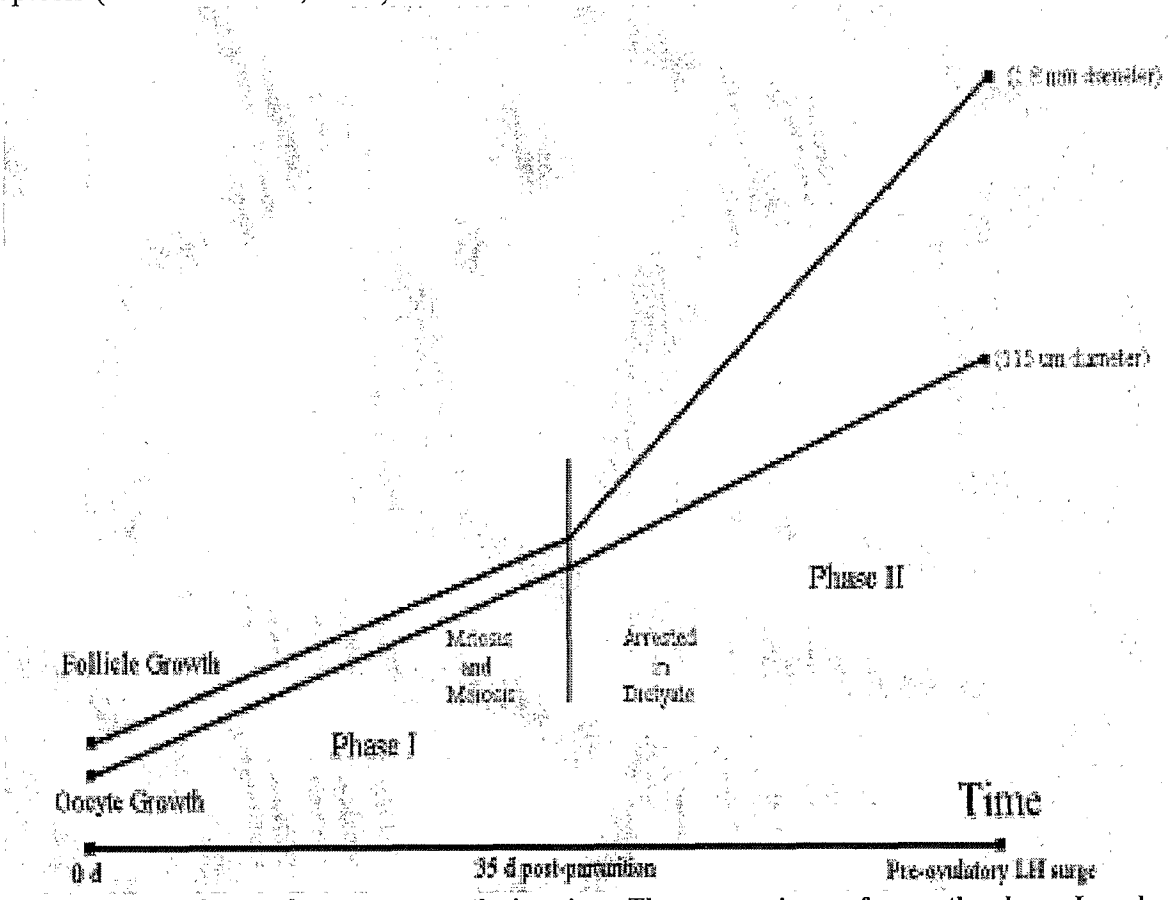

Figure 2.1. Follicular and oocyte growth in vivo. The comparison of growth phase I and growth phase II between the follicle and the oocyte.

The granulosa cells also act as paracrine centers for the oocytes and follicles by producing estrogen and other proliferative factors. Cumulus expansion is a result of synthesis and accumulation of the extracellular matrix component, hyaluronan (Kimura et al., 2002). The oocytes secrete cumulus-expansion enabling factor that helps regulate cumulus cell expansion via FSH or EGF which is mediated by cyclic adenosine monophosphate (cAMP) (Singh et al., 1993; Coskun et al., 1995). Vaderhyden (1993) demonstrated by measuring cumulus cell size expansion under the influence of FSH alone and FSH with the oocyte, that cumulus-expansion-enabling factor is not required for cumulus expansion. As the follicle and oocyte mature, the number of binding sites for FSH