Factors affecting in vitro maturation of alpaca (*Lama paco*) oocytes

THESIS

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Abstract

This study investigated factors that might affect in vitro maturation of alpaca oocytes. This resulted in a 2 x 2 x 2 factorial design having age (old vs. young), oocyte collection method (aspiration vs. slicing) and media supplementation (with or without fetal bovine serum [FBS]) as the independent factors utilized to determine optimum maturation rates. We hypothesized that oocytes aspirated from young alpacas and incubated in maturation media supplemented with FBS would have greater maturation rates than those incubated in any other factorial combination. Oocytes were collected from the ovaries of 11 young alpacas (≤ 10 years old) and 14 old alpacas (> 11 years old). Oocytes were aspirated from all follicles ≥ 2 mm on the ovarian surface using a needle and syringe. Once aspiration was completed, a scalpel blade was used to recover oocytes from all follicles ≤ 1 mm diameter by sequential slicing of the ovarian cortex. Oocytes recovered were classified as morphologically normal oocytes (MNO) and deemed suitable for incubation if ≥ 3 compact layers of cumulus cells and a homogeneous, evenly granulated cytoplasm were observed. MNO from each collection method were kept separate and halves of each group were randomly divided and incubated 24 hours in chemically defined maturation media with or without 10% FBS. Maturation was defined as the visualization of a polar body at the end of the incubation period. Of the total oocytes collected 37.5% were classified as MNO (298/794). Overall, aspiration yielded a greater percent of MNO than those obtained following slicing, 55% (136/247) versus

29.6% (162/547), respectively ($P < 0.05$). A greater percent of MNO reached maturation when collected by aspiration 36% (49/136) compared with slicing 8% (13/162) ($P < 0.05$). For MNO obtained by aspiration, a greater percent reached maturation when incubated in media supplemented with FBS compared with those incubated without FBS; 48.4% (32/66) versus 24% (17/70), respectively ($P < 0.05$). This study demonstrates that although ovarian slicing yields more total oocytes, a greater proportion of MNO are collected via aspiration of antral follicles ≥ 2 mm, and these MNO achieve greater maturation rates than MNO obtained from follicles ≤ 1 mm via ovarian slicing. Moreover, the addition of FBS to the incubation media improves maturation rates of oocytes obtained from antral follicles ≥ 2 mm, especially if collected from old alpacas.

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Fields of Study

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Chapter 1: Introduction

South American camelids (SAC) are an important livestock species in South America utilized for their fiber and meat. In addition, they have gained popularity in North America as companion animals and livestock as well. Considering their importance as a domesticated livestock species, current knowledge of their reproductive physiology is far behind that of other domestic species, although considerable advancements have been made in the last 20 years.

The validation and commercial application of assisted reproductive techniques in SAC is in its infancy. While embryo collection and nonsurgical transfer have been commercially adapted across the world, ovulation and embryo collection rate remains highly variable. In vitro technologies have gained popularity in research including oocyte maturation and embryo production, intracytoplasmic sperm injection and somatic cell nuclear transfer. To date the birth of a live offspring has yet to be documented from the transfer of an in vitro produced embryo from any one of these technologies in SAC.

Assisted reproductive techniques utilizing in vitro systems may be the future of SAC reproduction as it is for many other domestic species. Ovum pickup and in vitro embryo production holds the potential to produce more embryos with more pregnancies than conventional embryo collection and transfer (Mapletoft and Hasler 2005, Pontes et al. 2009). Employing in vitro technologies will assist in efficiently propagating

genetically superior animals. In order for this technology to gain commercial use in SAC, refinement of the in vitro culture system for oocyte maturation and embryo production is necessary. To date there have been no peer-reviewed reports published on in vitro maturation of immature alpaca oocytes.

Chapter 2: Review of Literature

New World camelids are comprised of two domesticated species the llama (*Lama glama*) and the alpaca (*Lama paco*) and two wild species the guanaco (*Lama guanicoe*) and the vicuna (*Lama vicugna*) (Bravo 2002). The llama and alpaca were first domesticated for meat and fiber production and are now utilized worldwide for this purpose as well as companion animals and packers (Bravo 2002). The guanaco and vicuna remain wild and are hunted for their meat and fine fiber, although recent attempts have been made to domesticate the vicuna. Old World camelids are comprised of the one humped dromedary camel (*Camelus dromedaries*) and the two humped Bactrian camel (*Camelus bactrianus*) (Skidmore et al. 1999). All four species of *Lama*, also known as South American camelids (SAC), and both species of *Camelus* can successfully interbreed resulting in fertile offspring (Skidmore et al. 1999). There have been reports of successful interbreeding between New World and Old World camelids employing artificial insemination. One of these crosses utilized semen collected from a male camel to inseminate a female guanaco which resulted in the live birth of a sterile hybrid offspring (Skidmore et al. 1999). Another cross from the same study again utilized semen collected from a male camel and inseminated a female llama resulting in the successful live birth of again a sterile hybrid offspring (Skidmore et al. 1999). Despite the reported success of utilizing artificial insemination in this study, the technique is not

widely employed in SAC reproduction due to the unique reproductive biology of the species and certain breed registry restrictions.

Unique aspects of camelid biology

Camelids are unique animals with several biologic features specific to them. They have a thickly padded foot rather than a hoof (Bravo 2002). Although they do ruminate, camelids are not classified as ruminants (Abbas et al. 1995). Their stomach is comprised of three digestive compartments which differ from the anatomy of true ruminants whose stomachs have four digestive compartments (Abbas et al. 1995). The dental pattern of the camelid also differs from that of true ruminants in that camelids have incisors and canine teeth on the top mandible (Abbas et al. 1995). Camelids can lose up to 30% of their body weight in water without any adverse effects (Gaughan 2011). Comparatively, most mammals cannot survive if they experience a loss of more than 12% (Gaughan 2011). Camelids can survive this extreme water loss due to the elliptical shape of their erythrocyte termed elliptocytes (Gaughan 2011, Omorphos et al. 1989). This distinctive shape allows the erythrocyte to withstand osmotic changes without lysing during periods of excessive water loss (Gaughan 2011, Omorphos et al. 1989). In addition the erythrocyte turnover decreases from 90 days in the winter to 150 days in the summer when the water supply is limited (Gaughan 2011).

Reproductive physiology

Most notably, camelids have a unique reproductive physiology. A broader knowledge exists regarding the reproductive physiology of the domesticated llama and alpaca. Distinct differences exist in the female reproductive anatomy as compared to true

ruminants in that SAC have two ovaries surrounded by a large ovarian bursa and the absence of intercornual ligaments (Tibary et al. 2007). In addition, the utero-tubal junction possesses well-developed papillae, and there is a distinct lack of multiple cartilaginous cervical rings (Tibary et al. 2007).

Puberty in the female can be defined by several criteria; age at first estrus, age at first ovulation, and age at which a female can support a pregnancy without deleterious effects (Bravo 2002, Senger 2005, Tibary et al. 2007). The precise onset of puberty in SAC is difficult to define as they are induced ovulators (Bravo 2002). Information on ovarian follicular activity obtained by urinary estrone sulfate indicates that follicular growth begins between 5 to 6 months of age (Bravo 1997). However, sexual receptivity is absent until SAC reach 12 to 13 months of age (Bravo 2002). In addition, SAC do not display regular estrous cycles exhibiting signs of overt estrus in the manner of spontaneously ovulating species (Bravo 2002, Vaughan 2011). The time of the first ovulation will depend on the age of the female at first mating as ovulation in the female SAC is induced by a 14 kDa protein in the seminal plasma of the male SAC which has recently been identified as β -nerve growth factor (Kershaw-Young et al. 2012_a). Domestic SAC are generally mated when they have reached two thirds of their mature body weight, which occurs at approximately 12 months in alpacas and 18 months in llamas (Senger 2005, Vaughan 2011).

SAC are considered non-seasonal breeders as ovarian follicular activity occurs throughout the year and season has no effect on the number of follicles observed on the ovary at any time and appearances of seasonality are largely due to nutrition and

management practices (Tibary et al. 2007, Vaughan 2011). The mammalian estrous cycle is divided into the follicular and luteal phase that assists in defining ovarian dynamics (Senger 2005). The SAC reproductive cycle differs from the typical mammalian cycle where the follicular phase is immediately followed by the luteal phase. SAC that have not been mated will demonstrate follicular waves that are the continuous renewing of terminally growing follicles (Vaughan 2011). The dynamics of antral follicles, characterized by the presence of an antrum filled with follicular fluid, as part of the follicular wave involve four processes: recruitment, selection, dominance and atresia or ovulation (Hennet and Combelles 2013, Senger 2005, Vaughan 2011). Recruitment is the phase of follicular development in which a group of small antral follicles approximately 2 to 3 mm in diameter called a cohort begins to grow and produce estradiol (Senger 2005, Vaughan 2011). Some of the recruited follicles undergo atresia, but a group of 1 to 3 follicles that have not undergone atresia, are then selected and continue to grow up to 3 to 5 mm (Senger 2005, Vaughan 2011). In SAC, as in other monotocous species, a single follicle will then become dominant, continue to grow and may undergo atresia or ovulation (Senger 2005, Vaughan 2004). Ovulations can result from dominant follicles ranging in size from 6 to 13 mm (Vaughan 2004). In SAC, the number of antral follicles on the ovaries as detected by ultrasonography is inversely proportional to the diameter of the largest follicle present (Vaughan et al. 2004). The entire duration of follicular growth is unknown in SAC, but is greater than follicular wave length, because the first stages of follicular growth are difficult to accurately assess with ultrasonography and are not included in the total estimation of the follicular wave

(Vaughan 2011). For this purpose, after new follicle emergence at the beginning of a follicular wave, follicle growth in SAC may be divided into three phases; growth, maturation, and regression (Vaughan 2011). The duration of the growth phase of the follicle in alpacas and llamas is approximately 5 to 9 days (Vaughan 2011). The follicle reaches a pre-ovulatory size of 6 to 12 mm in the mature phase, which lasts approximately 2 to 8 days followed by the regression phase that takes 3 to 8 days (Vaughan 2011). The interval between emergence of successive dominant follicles is known as the inter-wave interval and inter-wave interval varies widely among camelid species so generalizations should be avoided (Vaughan 2011). The average inter-wave interval is 10 to 22 days in llamas and alpacas, with the average follicular wave in alpacas occurring approximately every 15 days (Vaughan 2011, Bravo 2002).

SAC females that have not been mated and are nonovulatory are receptive to males most of the time regardless of ovarian follicular development (Bravo 2002). Female camelids assume the position of sternal recumbency for mating that lasts an average of 15 to 20 minutes, and copulation periods greater than 5 minutes have been sufficient to stimulate ovulation (Bravo 2002, Vaughan 2011). However, the likelihood of pregnancy resulting from copulation is highly dependent on the phase of follicular development with the maturation phase being the best for successful ovulation and conception (Bravo 2002, Vaughan 2011). Ovulatory sized follicles are those ranging from 7 to 12 mm, and the stimuli to elicit ovulation may include copulation, the administration of a hormone with luteinizing activity, and other types of stimuli such as a vasectomized male (Bravo 2002, Vaughan 2011). Ovulation after copulation or hormone

administration will occur as early as 24 hours and may take up to 3 days. Approximately 70 to 80% of camelid females will ovulate in response to appropriate stimuli with ovulation occurring equally on the right and left ovary (Bravo 2002, Vaughan and Tibary 2006). It has been reported that 5% of SAC, with a range as large 3.5 to 40%, will spontaneously ovulate a dominant follicle without any stimuli (Bravo 2002, Vaughan 2004, Vaughan and Tibary 2006).

Assisted reproductive techniques

Assisted reproductive techniques (ART) have been applied to varying extents in SAC. Multiple ovulation and embryo transfer (MOET) is a validated and commercially applied ART for SAC. Other ART such as artificial insemination (AI), in vitro oocyte maturation and in vitro embryo production require significant enhancements before they can be practically applied in SAC as they are in other domestic species such as horses and cattle. The unique reproductive biology of SAC has played a major role in the slow adaptation of ART to this species. However, just as in other domestic species, future genetic improvement in SAC will require the refinement of ART such as estrus synchronization, ovarian superstimulation, MOET and oocyte collection combined with in vitro fertilization (Vaughan 2004). This is due in large part to the fact that the generation interval in SAC is relatively long compared to other species because males reach puberty between 2 to 3 years and females exhibit an extended gestation between 11 to 12 months (Vaughan 2011, Bravo 2002). Compared to the generation interval in other livestock species such as cattle where males reach puberty at 7 to 18 months and gestation in the female lasts 9 months (Senger 2005).

Artificial insemination is a technique difficult to develop in SAC due to the protocols required for collecting, handling, extending and preserving semen (Bravo 2002). The first report of AI in SAC occurred 30 years ago, and although recent advances have been made in semen collection and preservation in the last 10 to 15 years, to date widespread commercial application of AI in SAC does not exist (Bravo 2002, Bravo et al. 2013). Difficulties in the collecting, handling, and processing of SAC semen arise due to the long copulation time averaging 15 to 20 minutes required by the male for a complete ejaculation, in addition to the extreme viscosity and oscillatory movement of the sperm without progressive semen motility (Bravo 2002, Giuliano et al. 2012, Kershaw-Young and Maxwell 2012_c, Vaughan 2011). Various collection methods have historically included vaginal sacs and vaginal sponges beginning in 1952 for alpacas and 1989 for llamas (Bravo 2002). More recently, the use of artificial vaginas, recovery from the vagina of a mated female, and electro-ejaculation are among the most common methods currently employed to collect semen (Guiliano et al. 2008, Bravo 2002). Recent investigation into the composition of SAC semen has proven that semen viscosity is due to the high concentration of glycosaminoglycans (GAGs) present in the seminal plasma with the bulbourethral gland being the major contributing sex gland of GAGs to the seminal plasma (Kershaw-Young et al. 2012_b). Keratin sulfate is the GAG present in the highest amount and this GAG was shown to be correlated with viscosity in SAC semen (Kershaw-Young et al. 2012_b). Liquefaction of the viscous semen is necessary for the evaluation and processing necessary for AI (Bravo et al. 2000). Liquefaction of SAC will occur naturally but may take several hours (Bravo et al. 2000). Liquefaction can be

artificially induced without detrimental effects to sperm by the use of a hydrolytic and proteolytic enzyme such as collagenase, hyaluronidase, trypsin, or fibrolysin (Bravo et al. 2000, Tibary et al. 2007). Few studies have been conducted to find the best extenders for the different utilization of alpaca semen including fresh dilution, refrigeration, and cryopreservation (Bravo et al. 2013). Extenders investigated include tris-buffered, milk based, and egg-yolk based, and although tris-buffered extenders improve semen motility to date no significant differences exist between extender composition and pregnancy rate (Bravo et al. 2013, Miragaya et al. 2005).

Embryo transfer was first reported by Peruvian researchers in alpacas in 1968 (Novoa and Sumar 1968). Since then this technique has been utilized conservatively in SAC with limited success compared to other domestic species such as cattle and horses where this technique is routinely employed with success. Historically, superovulation of SAC was relatively unsuccessful; therefore common practice was to collect single embryos from a donor during several consecutive reproductive cycles (Sutmoller 1999). However, in the last 10 to 15 years superovulation has been investigated and commercially utilized in countries with high populations of SAC (Tibary et al. 2005). Limited information is available on early embryonic development and optimum day for embryo collection in SAC (Vaughan et al. 2013). It is known that the embryo in SAC enters the uterus 5 to 6 days after ovulation which equates to 6 to 7 days post mating (Tibary et al. 2007). Embryo collection can occur any time after the embryo enters the uterus 5 to 6 days after ovulation and prior to embryonic recognition of pregnancy on day 9 (Tibary et al. 2007, Vaughan et al. 2013). Typically, embryo collection is performed 7

to 8 days after ovulation with great variation in embryo recovery rates influenced by factors such as superovulation protocol, age, parity, nutrition status, collection day, and technician experience (Tibary et al. 2007, Vaughan et al. 2013). Nonsurgical and surgical methods have been employed to perform embryo collection and transfer in SAC. Surgically, midline laparoscopy and laparotomy have been successfully used to recover and transfer embryos (Sansinena et al. 2003, Sumar 2013, Tibary et al. 2007). Recently, nonsurgical techniques have been refined to allow the collection and transfer of SAC embryos with or without rectal manipulation of the reproductive tract (Vaughan et al. 2013, Coutinho da Silva et al. 2012). SAC embryos are hatched blastocyst stage embryos at the time of collection; this is distinctively different from ruminant embryos that are at the blastocyst stage at the time of collection. The blastocyst develops from the cavitation and polarization of a morula, described as a ball of embryonic cells called blastomeres, resulting in the formation of a fluid filled cavity called the blastocoele cavity and two distinctive cell types (Senger 2005). These two cell types are: the trophoblast, which forms the chorion and the inner cell mass, which forms the embryo proper (Senger 2005). Furthermore, a hatched blastocyst is no longer surrounded by the zona pellucida (ZP), a translucent membrane comprised of glycoproteins that plays a critical role in fertilization (Gupta et al. 2012). Hatched blastocyst embryos collected from SAC can range in size from 400 to 2500 microns depending on the day of collection (Sutmoller 1999, Tibary et al. 1997). The size of the SAC embryo and the absence of the ZP have made cryopreservation of these embryos difficult and few studies have been conducted to find the best protocol for this species (Aller et al. 2002, Tibary et al. 2007). Recent success

has been reported achieving a 50% pregnancy rate with llama embryos, collected from superovulated donors, cryopreserved by vitrification, and nonsurgically transferred to llama recipients (Aller et al. 2002). Factors hindering the widespread commercial use of embryo transfer are the restrictive rules and regulations of the breed registries and associations in the United States of America. Many registries are not in favor of the use of embryo transfer and will not allow offspring to be registered if such techniques have been employed to produce them.

In many species such as cattle, in vitro oocyte maturation and subsequent embryo production is commonly applied and gaining popularity. In 2011 approximately 38% of the world's bovine embryos were produced by in vitro technology (Stroud 2012). In vitro production of bovine embryos has the potential to produce more embryos and pregnancies compared to conventional MOET (Mapletoft and Hasler 2005, Pontes et al. 2009). The development of an in vitro embryo production system for SAC may be a useful tool for the genetic improvement of this species (Sansinena et al. 2007). In addition, in vitro embryo production may overcome some limitations associated with embryo transfer in SAC such as the variability in response when using superovulation and embryo cryopreservation. Embryos recovered from the uterus are extremely difficult to cryopreserve because they are hatched blastocysts. In vitro produced embryos could then be cryopreserved prior to blastocyst expansion and hatching from the ZP (Berland et al. 2011). This area of ART in SAC has begun to receive more attention over the last decade. However, the published reports on in vitro embryo production involve work conducted primarily in llamas, with very few reports involving alpacas.

Collection of SAC oocytes can be difficult and several techniques have been applied to collect oocytes from follicle aspiration. The methods available for ovum pickup from SAC including a midline or flank laparotomy, laparoscopy, and transvaginal ultrasound-guided aspiration (Berland et al. 2011, Brogliatti et al. 2000, Ratto et al. 2007, Sansinena et al. 2007, Trasorras et al. 2009). Flank laparotomy results in an 80% recovery rate, the highest of any technique (Sansinena et al. 2007). However, laparoscopic ovum pick up is safer with relatively similar recovery rates (Sansinena et al. 2007, Tibary et al. 2007). Transvaginal ultrasound-guided aspiration is a relatively noninvasive, repeatable procedure, but it has the lowest recovery rates ranging from 30 to 75% and may be physically impossible in animals of smaller stature, limiting the use of this technique in alpacas (Brogliatti et al. 2000 Tibary et al. 2005, 2007).

The first successful in vitro maturation of SAC oocytes was achieved in the llama in 1992 followed by the first in vitro production of embryos, again in the llama in 1994 (Del Campo et al. 1994). To date the birth of a llama or alpaca from the transfer of an in vitro produced embryo has not been documented (Berland et al. 2011). However, limited success has been achieved utilizing in vitro technologies in Old World camelids. The birth of live offspring has been reported in the dromedary camel from the transfer of in vitro produced embryos derived from camel oocytes matured and fertilized in vitro (Khatir and Anouassi 2006). In addition, somatic cell nuclear transfer and in vitro embryo culture resulted in the birth of the first cloned dromedary camel calf (Wani et al. 2009). However, the refinement of oocyte maturation, fertilization, and embryo culture

will be necessary to attain success utilizing in vitro techniques in SAC (Berland et al. 2011).

Recently, ovarian superstimulation in conjunction with oocyte recovery has been investigated to eliminate the variability associated with superovulation and embryo collection (Berland et al. 2011). Successful in vitro production of embryos is possible when oocytes are aspirated from superstimulated ovaries of SAC. The technique of ovarian superstimulation using equine chorionic gonadotropin or follicle stimulating hormone (FSH) followed by oocyte aspiration provides a uniform population of oocytes ready for collection, which have matured in vivo (Ratto et al. 2005, Tibary et al. 2007, Trasorras et al. 2007). Blastocyst rates (defined as the number of blastocysts produced from inseminated oocytes) as low as 15% and as high as 21% have been seen using oocytes recovered from superstimulated llamas and fertilized in vitro with llama semen (Berland et al. 2011, Trasorras et al. 2012).

Additional technologies have been applied to SAC oocytes collected from superstimulated llamas in an attempt to aid in vitro embryo production with limited success. These technologies include somatic cell nuclear transfer and intracytoplasmic sperm injection (ICSI). Utilizing ICSI eliminates the necessity of collecting a good quality ejaculate with adequate concentration, which can pose a problem in SAC (Sansinena et al. 2007). Embryo production rates using ICSI with llama oocytes and llama sperm has ranged as low as 9.6% from oocyte donors that were not superstimulated to 12% from oocyte donors that were superstimulated (Del Campo et al. 1994, Sansinena et al. 2007). These studies emphasize that a major constraint to proper embryo

development is oocyte maturation. Few studies have investigated in vitro factors that affect oocyte maturation in SAC and even less in alpacas specifically.

In vitro oocyte maturation

Final growth, capacitation, and maturation of the oocyte occur in the last few days preceding ovulation (Hennet and Combelles 2013). The follicular microenvironment is critical to oocyte competence, which is defined as the potential to undergo maturation, fertilization, and development to blastocyst stage (Jiao and Woodruff 2012). A greater understanding of the follicular microenvironment will assist in providing a suitable microenvironment for in vitro oocyte maturation (Hennet and Combelles 2013). The follicular fluid of an antral follicle is derived from the bloodstream and components from somatic cells within the developing follicle (Hennet and Combelles 2013). This rich follicular fluid comprised of steroid and protein hormones, growth factors, enzymes, anticoagulants, and electrolytes encompasses the developing oocyte, mediating its growth and communication between the oocyte and surrounding cells (Hennet and Combelles 2013).

The cell types found in a developing antral follicle are the theca interna and granulosa cells (Senger 2005). The theca cells are the outer margin of the developing antral follicle (Hennet and Combelles 2013, Senger 2005). The granulosa cells can be further divided into the mural granulosa which form the internal lining of the follicle and cumulus cells, which surround the oocyte and form what is known as the cumulus-oocyte complex (COC) (Del Campo et al. 1995b, Hennet and Combelles 2013, Rienzi et al. 2012). The innermost cumulus cells that surround the oocyte itself are called the corona

radiata (Rienzi et al. 2012). The cumulus cells are a specialized type of granulosa cell and play an integral role in oocyte development and maturation (Hennet and Combelles 2013). The COC protrudes into the antrum of the follicle and is attached to the follicular wall by a neck of granulosa cells (Del Campo et al. 1995_b). During the final hours of follicular and oocyte maturation the cumulus cells are involved in the release of the oocyte from attachment to the follicular wall by expansion and loosening so ovulation of a competent oocyte can occur (Del Campo et al. 1995_b). The follicles of SAC, as studied in llamas, have a distinctively spherical shape with 91% of the COC located in the ovulation hemisphere, defined as the half of the follicular sphere with the ovulation site at its pole (Del Campo et al. 1995_b). Polyovular follicles are rare in SAC but have been reported (Del Campo et al. 1995_b).

The ultrastructure of the SAC oocyte has similarities to oocytes of other domestic species such as cattle and horses (Brogliatti et al. 2000, Del Campo et al. 1995_b). The cumulus cells are tightly attached to the ZP with abundant cellular projections that penetrate the ZP and connect with the oocyte at either the ZP or more often at invaginations within the oolemma (Brogliatti et al. 2000). The perivitelline space, the space between the ZP and the ooplasm, is not apparent in immature oocytes collected from SAC (Brogliatti et al. 2000). However, the perivitelline space does increase as oocyte maturation progresses up to 24 hours and then the size of the perivitelline space is maintained until 36 hours (Tibary et al. 2007). A distinctive feature of SAC oocytes is the presence of a high number of lipid droplets in the ooplasm that migrate throughout the ooplasm during in vitro maturation but do not decrease in numbers or size (Brogliatti

et al. 2000). This high content of lipid droplets may attribute to the dark appearance of the oocyte cytoplasm found in SAC (Brogliatti et al. 2000).

In human and domestic species in general, current in vitro fertilization protocols fail to fully support optimum oocyte maturation and embryo production. The limitation of these protocols is that they do not fully replace the components involved in follicle and oocyte maturation in vivo (Hennet and Combelles 2013). It is imperative that these protocols maintain oocyte health and function to promote growth and development, allow cumulus cell proliferation to prevent the oocyte from becoming denuded, and maintain proper cumulus cell associations during culture (Hirao 2011). An appropriate in vitro fertilization protocol must provide conditions for proper oocyte maturation and competence, allowing for fertilization and the development of a viable embryo (Hirao 2011, Jiao and Woodruff 2012). A visual indication of oocyte nuclear maturation is the extrusion of the first polar body (PB) from the ooplasm into the perivitelline space of the female oocyte (Rienzi et al. 2012). Two PB eventually form once an oocyte has undergone fertilization. This is because the meiotic divisions are unequal in female meiosis and the first PB is extruded after the resumption of meiosis I in preparation for fertilization (Jiao and Woodruff 2012). The second PB is extruded after fertilization and the resumption of meiosis II (Jiao and Woodruff 2012).

Studies have evaluated in vitro maturation of SAC oocytes by assessing PB extrusion in llamas. The first reports of in vitro llama oocyte maturation reported 62% nuclear maturation with 36 hour incubation, followed by a second study incubating for 30 hours reporting a 30.4% maturation rate (Del Campo et al. 1994). A 36-hour incubation

time correlates to the approximate event of oocyte maturation and ovulation after mating in SAC (Ratto et al. 2005). Another study utilizing a 30-hour incubation time reported a 52% in vitro maturation rate in llama oocytes (Sansinena et al. 2003). However, it has been shown that in vitro maturation in llama oocytes can be seen as early as 24 hours of incubation (Brogliatti et al. 2000). The most recent study evaluating in vitro maturation in llamas demonstrated that adequate maturation rates near 80% could be achieved with various incubation times. In vitro maturation rates of 78%, 81%, and 80% were achieved in oocytes recovered by aspiration and incubated for 28, 30, and 36 hours respectively; these are the highest in vitro maturation rates reported in SAC (Ratto et al. 2005).

Several factors may contribute to oocyte maturation and these factors include but are not limited to follicle size, animal age, and media composition. In llamas, 62% of COC reached maturation when they were aspirated from follicles 1 to 12 mm in size with an average recovery rate of 6 oocytes per female (Del Campo et al. 1995_a). This is compared to ovarian mincing, which recovers 27 oocytes per female from follicles of various sizes, but results in significantly lower maturation rates at 30.4% (Del Campo et al. 1994, 1995_a).

In other species such as mice and horses, age has been shown to cause a significant decrease in oocyte competence (Brinsko et al. 1995, Jiao et al. 2012). This increase in oocyte age resulted in a 70.5% blastocyst rate from aged mouse oocytes versus 82.8% from young mouse oocytes (Jiao et al. 2012, Jiao and Woodruff 2012). In the horse, oocytes collected from old animals were only 25% likely to reach in vitro maturation and were three times more likely to remain immature than those collected

from young or middle aged animals (Brinsko et al. 1995). It has also been shown that oocyte specific mRNA expression decreases in oocytes from aged mice, thus accounting for reduced competence of aged oocytes (Jiao and Woodruff 2012). Aged oocytes have been shown to elicit functional changes in the oolemma, ZP, cytoskeletal arrangement, decreased fertilization rates, polyspermy (Longo 1981, Webb et al. 1986, Wortzman and Evans 2004). Many failures in ART are associated with oocyte age; therefore technologies should be utilized to prevent failures or abnormalities associated with the use of aged oocytes in ART systems (Miao et al. 2009).

In vitro culture media

To date the use of serum supplemented culture media remains the most efficient method for culturing cells in vitro (Broussard et al. 2010). Although serum-supplemented media yields the best culture results, the composite of hormones, nutrients, and growth factors in serum remains largely undefined. It has been shown that serum-supplemented media can adversely affect the speed of embryo development and resulting blastocyst quality in bovine embryos (Rizos et al. 2003). In addition, bovine blastocysts produced in serum-supplemented media display deviations in the abundance of developmentally important gene transcripts and a reduced cryotolerance (Oliveira et al. 2012, Rizos et al. 2003). Recently, the development of chemically defined culture media to conduct in vitro fertilization in domestic species has received more attention due to biosecurity and storage concerns related to the use of animal serum (Hasler 2010). To date, only one study in SAC has utilized a defined media system to produce in vitro llama embryos with a 20% blastocyst production rate and 9% hatched blastocyst rate on day 6

using synthetic oviductal fluid media (Trasorras et al. 2012). This blastocyst rate is comparable to the 21% rate achieved using somatic cell co-culture (Berland et al. 2011, Trasorras et al. 2012). Compared to a previously reported 4.7% in vitro blastocyst hatching rate achieved with somatic cell co-culture in llamas, the 9% hatching rate achieved with defined media is the highest ever reported (Del Campo et al. 1994, Trasorras et al. 2012). In addition to biosecurity concerns inherent to the use of animal serum, defined media is easier to prepare and presents a much lower risk of contamination than the preparation of somatic cell monolayers used for co-culture (Trasorras et al. 2012).

The combination of factors such as media composition, follicle size and animal age can have a profound impact on in vitro oocyte maturation rates and to our knowledge has not previously been investigated in SAC, particularly in alpacas. As a result, the present study investigated the effects of animal age (young vs. old), follicle size (≥ 2 mm vs. ≤ 1 mm), and in vitro maturation media supplementation (with or without FBS) on in vitro maturation of alpaca oocytes. We hypothesized that oocytes collected from young alpacas by aspiration of follicles ≥ 2 mm and incubated in media supplemented with FBS would have the highest maturation rates.

Chapter 3: Materials and Methods

Animals

All animals were housed and handled at the Ohio State University Veterinary Medical Center according to an approved Institutional Animal Care and Use Committee protocol. Groups of ≤ 6 female alpacas were housed separate from male alpacas in 12 foot by 10-foot stalls with access to water ad libitum and fed free choice grass hay. A total of 25 alpacas were used in the study. According to the classification of geriatric alpacas as defined by Bravo (2002), alpacas enrolled in the present study were divided into two experimental groups: young ≤ 10 years old (n=11) and old > 11 years old (n=14).

Experimental design and treatments

A 2 x 2 x 2 factorial design was used to evaluate the independent effects of alpaca age (young vs. old), collection method (aspiration vs. slicing), and media supplementation (with or without FBS) on in vitro alpaca oocyte maturation (Figure 1).

Figure 1: Experimental design

Reproductive examinations per rectum utilizing an ultrasound system (Aloka, model: SSD-500, Hitachi Aloka ©, Wallingford, CT) equipped with a 7.5 MHz linear transducer (model: UST-660-7.5 Hitachi Aloka ©, Wallingford, CT) were performed on all alpacas prior to study enrollment. Ultrasonography confirmed non pregnant status of all alpacas and ruled out major reproductive pathologies.

Alpacas were euthanized with 110 mg/kg of euthanasia solution (MWI, Meridian, ID). Ovaries were harvested immediately following euthanasia (Figure 2). A midline incision was made immediately cranial to the mammary glands with a 22-scalpel blade, and the reproductive tract was located. The pair of ovaries was removed using scissors and placed in 50-mL conical tubes with 25 mL sterile 0.01 M phosphate buffered saline (PBS) (P3813, Sigma-Aldrich®, St. Louis, MO) warmed to 37°C. Conical tubes were placed in a tube rack inside a small Styrofoam box with a bag filled with 1 liter of tap

water warmed to 37°C for transport to the laboratory. Once all ovaries were collected and transported to the laboratory, each pair was kept separate and rinsed twice with sterile PBS warmed to 37°C. After rinsing each pair was placed in a 60-mm Petri dish with 3 mL PBS warmed to 37°C.

Figure 2: Ovary collection

Ovaries were aspirated following rinsing with PBS (Figure 3). All follicles ≥ 2 mm were aspirated using a 10-mL all-plastic syringe coupled with a 20-gauge 1-inch needle and the number of follicles aspirated was recorded. Prior to aspiration the needle and syringe were prefilled with 1 mL of chemically defined oocyte handling media (HCDM-M; Appendix A) (De La Torre-Sanchez et al. 2006) warmed to 37°C. When ovary aspiration was completed on one ovary, that syringe was emptied into a 10-mm

Petri dish filled with 2 mL of HCDM-M at 37°C and held on a warm plate until oocyte searching could be conducted. Each aspirated ovary was emptied into a separate dish to track follicle aspiration and oocyte recovery. After aspiration, ovary pairs from respective alpacas were sliced in a 60-mm Petri dish with 2 mL 37°C PBS and 2 mL 37°C HCDM-M. Ovaries were held in place with an 18-gauge 1-inch needle and minced with a 22-scalpel blade by sequential slicing of the ovarian cortex (Figure 3). When slicing was completed, ovaries were rinsed with 2 mL of HCDM-M using a 5-mL all-plastic syringe and an 18-gauge 1-inch needle, and then removed from the dish and discarded.

Figure 3: Ovary aspiration and slicing for oocyte collection

Dishes containing aspirated and sliced oocytes from each alpaca were kept separate. Oocytes were identified with a stereomicroscope (SMZ1500, Nikon

Instruments Inc, Melville, NY) and handled with a 1-mL tuberculin syringe fitted with a 3.0 to 6.0 French ureteral catheter connector (050010, Cook Medical©, Bloomington, IN) and 10- μ L glass capillary tube (19022/1100, Minitube of America®, Verona, WI). All visualized oocytes were retrieved and placed in a 10-mm Petri dish with 2 mL fresh 37°C HCDM-M. Oocytes were classified using a stereomicroscope and only morphologically normal oocytes (MNO) were selected for in vitro incubation according to the following criteria; 1) the oocyte cytoplasm was homogeneous and evenly granulated 2) the oocyte was surrounded by a compact mass of cumulus cells with at least 3 layers of cells (Figure 4).

Figure 4: Morphologically normal oocytes

Oocytes recovered that were denuded, had a broken ZP, unevenly granulated cytoplasm, or surrounded with a fibrous mass of cumulus cells were discarded for incubation (Figure 5).

Figure 5: Alpaca oocytes unsuitable for incubation

Aspirated and sliced MNO from each alpaca were kept separate and washed three times through 10-mm Petri dishes with 2 mL of HCDM-M warmed to 37°C. Halves of each group of oocytes from each alpaca were placed into 4-well dishes (12-566-350, Nunc IVF, Fisher Scientific, Waltham, MA) with 1 mL of chemically defined in vitro maturation media (IVM; Appendix A) (De La Torre Sanchez et al. 2006) in each well with or without the addition of 10% heat inactivated FBS (166140-063, Invitrogen™, Grand Island, NY). See Figure 1 for arrangement of dish wells according to experimental design. Hormones were added on the day of maturation to all IVM media; FSH 15 ng/μl (national hormone and peptide program-NIDDK, UCLA, Los Angeles, CA), luteinizing hormone (LH) 1 μg/μl (national hormone and peptide program-NIDDK, UCLA, Los

Angeles, CA), epidermal growth factor 50 ng/ μ l (E9644, Sigma-Aldrich®, St. Louis, MO), Estradiol 17 β 0.1 μ g/ μ l (E2257, Sigma-Aldrich®, St. Louis, MO), and Cysteamine 10 mM (M6500, Sigma-Aldrich®, St. Louis, MO). MNO were handled with a 2 to 20 μ l pipette (Gilson Pipetman P-20, AccuTek Labs, San Diego, CA) and general-purpose pipette tip (701251, Reproduction Resources, Walworth, WI) and placed in IVM with $\leq$ 10 μ l HCDM-M. MNO were placed in culture within 1 to 1.5 hours from the time ovaries were collected. MNO were incubated at 38.5°C in 6% CO₂ in air and 100% humidity for 24 hours (Forma Series II Water Jacket CO₂ Incubator Model 3110, Fisher Scientific, Waltham, MA).

At 24 hours the expanded cumulus cells were removed from all of the incubated MNO, a process known as denuding, and all aspirated and sliced MNO incubated in the presence or absence of FBS from each animal were again kept separate (Figure 6).

Figure 6: Expanded cumulus cells of morphologically normal oocytes collected by aspiration or slicing, incubated for 24 hours in media with or without fetal bovine serum

Denuding of MNO was carried out by pipetting with the 2 to 20 μ L pipette and general-purpose tip when oocyte numbers were ≤ 5 per group. If oocyte numbers were ≥ 6 per group, oocytes were placed in a 0.5 mL micro centrifuge tube (05-408-120, Fisher Scientific, Waltham, MA) with 5 μ L IVM media and placed on a vortex mixer at speed 10 for 90 seconds (SN091006038, Fisher Scientific, Waltham, MA). The centrifuge tube was filled with 500 μ L IVM and allowed to settle for 60 seconds. The denuded oocytes were aspirated from the bottom of the centrifuge tube and returned to the appropriate well in the IVM dish. When denuding was completed, nuclear maturation was evaluated in each oocyte. Nuclear maturation was defined as the visualization of the first polar body (Figure 7) and was assessed using a stereomicroscope and an inverted microscope with Hoffman modulation contrast (Eclipse Ti, Nikon Instruments Inc, Melville, NY).

Figure 7: Mature alpaca oocyte with cumulus cells removed and polar body present (arrow)

Statistical Analysis

A multiple linear regression was used to analyze the effects of the independent variables on maturation rate. A Chi Square test (two-tailed) with Yates correction for continuity when appropriate, was used to evaluate individual specific factorial arrangements (Sigma Stat, Jandel Corp., San Rafael, CA). Data are expressed as mean $\pm$ SD. Significance was set at $P < 0.05$ with a trend for significance set at $0.05 < P < 0.1$.

Chapter 4: Results

From the 25 alpacas used in the study, 50 ovaries were collected. Four alpacas enrolled in the study were found to have ovarian abnormalities. Three alpacas, two old and one young, each had follicular cysts between 13 to 22 mm and these ovaries were still utilized for collection of immature oocytes. One young alpaca had an ovarian teratoma and as a result that ovary was discarded from the study. From the remaining 49 ovaries, a total of 794 oocytes were recovered, and 37.5% (298/794) of the total oocytes collected were classified as MNO. An average of $31.8 (\pm 26.8)$ oocytes was collected per alpaca, with $9.9 (\pm 10.6)$ collected by aspiration and $21.9 (\pm 18.9)$ collected by slicing. More oocytes were collected using the slicing method as compared with aspiration; 547 and 247, respectively. However, a greater proportion of MNO were collected by the aspiration method than the slicing method (Table 1). When evaluated by age, the proportion of MNO according to collection method remained different in both age groups with more MNO collected by aspiration than slicing (Table 1). In addition, there was a trend for a greater proportion of MNO to be collected by aspiration from young than from old alpacas (Table 1).

Table 1: Percentage of morphologically normal oocytes (MNO) obtained according to age and collection method

Source of MNO	Aspiration	Slicing
Total (Young + Old)	55.5% (136/247)*	29.6% (162/547)*
Young	61.4% (70/114)*#	30.1% (91/302)*
Old	49.6% (66/133)*#	29% (71/245)*

* Within rows denotes $P < 0.05$; # within columns denotes $0.05 < P < 0.1$

Two of the three factors investigated in this study, collection method and maturation media, resulted in a significant effect on percent maturation of MNO ($P < 0.05$). Overall, the maturation rate of all MNO collected in this study (old and young, aspirated and sliced) was 20.8% (62/298). A greater percentage of MNO reached nuclear maturation when collected by the aspiration method compared with those collected by slicing, regardless of age (Table 2).

Table 2: Percent maturation rate of morphologically normal oocytes (MNO) according to age and collection method

Source of MNO	Aspiration	Slicing
Total (Young + Old)	36% (49/136)*	8% (13/162)*
Young	34.3% (24/70)*	7.7% (7/91)*
Old	37.9% (25/66)*	8.5% (6/71)*

* Within rows denotes $P < 0.05$

All MNO collected by aspiration and incubated in media supplemented with FBS reached greater maturation rates than those collected by slicing and cultured in the same media (Table 3). When evaluated by age group, MNO collected by aspiration and incubated in media supplemented with FBS reached greater maturation rates than those collected by slicing in both young and old alpacas (Table 3). The MNO from both young and old alpacas collected by the aspiration method had greater percent maturation than those collected by slicing when incubated in media without the addition of FBS (Table 3). When stratified by age, MNO from young alpacas collected by aspiration and incubated in media without FBS reached greater maturation rates than those collected by slicing and incubated under the same conditions (Table 3). There was no difference in maturation rates between MNO collected by aspiration or slicing from old alpacas when incubated in media without FBS (Table 3).

Overall, MNO from young and old alpacas collected by aspiration and incubated in media supplemented with FBS reached greater maturation rates than those collected by the same method and incubated in media without FBS (Table 3). When evaluated by age, there was no difference in maturation rate of MNO from young alpacas collected by aspiration and incubated in media with FBS compared with those incubated without FBS (Table 3). In contrast, a greater percent of MNO collected by aspiration from old alpacas reached maturation when incubated in media supplemented with FBS compared with those incubated without FBS (Table 3). Overall and when evaluated by age, there was no difference in the maturation rate of MNO from young and old alpacas collected by the slicing method and incubated in media with or without FBS (Table 3).

Table 3: Percent maturation rate of morphologically normal oocytes (MNO) according to age (young vs. old), collection method (aspiration vs. slicing) and maturation media (with or without fetal bovine serum [FBS])

+ FBS		
Source of MNO	Aspiration	Slicing
Total (young + old)	48.5% (32/66) ^{* #}	6.1% (5/82) [*]
Young	39.4% (13/33) [*]	6.4% (3/47) [*]
Old	57.6% (19/33) ^{* #}	5.7% (2/35) [*]
- FBS		
Source of MNO	Aspiration	Slicing
Total (young + old)	24% (17/70) ^{* #}	10% (8/80) [*]
Young	29.7% (11/37) [*]	9.1% (4/44) [*]
Old	18.2% (6/33) [#]	11.1% (4/36)

^{*} Within rows and [#] within columns denotes $P < 0.05$

Chapter 5: Discussion

To our knowledge, this is the first study that evaluated the effects of age, collection method and media supplemented with or without FBS on in vitro maturation of alpaca oocytes. In fact, to date, there have been no peer-reviewed reports on in vitro maturation of immature alpaca oocytes. The factors investigated in the present study were found to have significant effects on oocyte recovery and maturation in alpacas with significant interactions observed between factors. Specifically age and collection method significantly affected the recovery of oocytes and the proportion of MNO; whereas, type of media and collection method affected the proportion of MNO that reached maturation after incubation.

Slicing of the ovarian cortex yielded a greater oocyte recovery than that obtained by aspiration. This finding is in agreement with other studies that found a greater oocyte recovery in llamas when collected by slicing as compared with aspiration (Del Campo et al. 1994). However, a greater percent of oocytes recovered from follicles ≥ 2 mm by aspiration as compared with slicing were classified as MNO. This difference in percent of MNO collected likely results from the fact that slicing recovers oocytes from the pool of follicles present on the ovary that may be at any stage of development including primordial, primary, secondary, and early antral follicles (Moniruzzaman and Miyano 2010). With slicing, oocytes at various developmental stages are collected from this pool

of follicles including oocytes with few to no cumulus cells, oocytes that are undergoing atresia and irreversible apoptotic changes, and oocytes in the resting state due to repressive signals within the ovary (Moniruzzaman and Miyano 2010, Tingen et al. 2009). Therefore, oocytes collected by slicing were less likely to be morphologically normal according to the criteria utilized in the present study.

There was a trend for a greater proportion of MNO to be recovered from aspiration in young than from old alpacas. This is likely due to the fact that females undergoing physiological reproductive aging experience a decrease in oocyte quality and developmental competence (Berlinguer et al. 2012, Malhi et al. 2007). In horses, sheep and cattle, it has been demonstrated that oocytes recovered from aged animals and deemed suitable for incubation were less likely to achieve maturation than those collected from young animals (Berlinguer et al. 2012, Brinsko et al. 1995). In sheep, the proportion of morphologically abnormal oocytes was significantly higher in aged animals (Berlinguer et al. 2012). Aged oocytes undergo several morphological changes that are deleterious to developmental competence. Some of these changes include structural changes of the plasma membrane, ZP hardening, displacement of the first PB and premature exocytosis of cortical granules (Miao 2009).

The design of the present study sought to evaluate the difference in maturation rates between oocytes collected from antral follicles ≥ 2 mm and follicles ≤ 1 mm. By aspirating follicles ≥ 2 mm followed by sequential slicing of the ovary to recover oocytes from follicles ≤ 1 mm, two distinct populations of follicles could be sampled to provide oocytes for this study. It became evident that although MNO can be recovered from

follicles ≤ 1 mm and may reach nuclear maturation, antral follicles ≥ 2 mm yielded more MNO that achieved greater maturation rates than MNO obtained from follicles ≤ 1 mm. Oocytes retrieved by follicular aspiration from antral follicles ≥ 2 mm may be more developmentally competent because virtually all oocytes recovered from these follicles are at a stage of follicle development in which they are enveloped in the COC. The presence of healthy cumulus cells is crucial for adequate oocyte maturation (Del Campo et al. 1994, Hennet and Combelles 2012, Hirao 2011, Rienzi et al. 2012). The bidirectional communication between the oocyte and the microvilli projections of the corona radiata, which cross the ZP and form gap junctions with the oolemma, is vital to the competence of the developing oocyte (Rienzi et al. 2012).

Oocytes recovered from follicles ≤ 1 mm have a longer growth and maturation phase and likely require different culture conditions than oocytes collected from antral follicles ≥ 2 mm (Moniruzzaman and Miyano 2010). This characteristic may have explained the lower maturation rates of oocytes recovered by slicing compared with aspiration when incubated under the same conditions. Moreover, a factor which may have negatively influenced the maturation rate of MNO collected by the slicing method compared to those collected by aspiration may have been the use of PBS in this study. All ovaries were sliced in PBS and HCDM-M in contrast to follicles that were aspirated directly into HCDM-M only. PBS is an isotonic solution with a physiological pH of 7.4 that has been used as a handling medium for oocytes and embryos (Swain and Pool 2012). In cattle and rabbits, it has been shown that oocytes handled in PBS for time periods of greater than 40 minutes resulted in reduced embryo production than other

buffered oocyte handling media (Escriba et al. 2001, Palasz et al. 2008). This is attributed to the fact that PBS lacks metabolic substrates and bicarbonate (Swain and Pool 2012). In mice it has been demonstrated that exposure to elevated levels of phosphate, the main component of PBS, will disrupt organelle distribution in the maturing oocyte, ultimately compromise embryo production and pregnancy rates (Barnett and Bavister 1996). The potentially damaging effects of PBS on oocyte maturation are not evident in every mammalian species. For example, in the domestic cat oocytes matured in vitro for embryo production have achieved adequate blastocyst rate when utilizing PBS as a medium (Kitiyanant et al. 2003). Potential deleterious effects of PBS on alpaca oocytes have not been determined. The experimental design of the present study did not account for the potential effects of the transient exposure (30 to 60 minutes) to PBS on MNO collected by slicing.

In the SAC, limited information is available regarding protocols for in vitro maturation of immature oocytes (Del Campo et al. 1994, 1995_a, Ratto et al. 2005, Sansinena et al. 2003, 2007, Trassoras 2009). However, no information is available on in vitro maturation of immature oocytes collected from alpacas. In the six studies conducted on in vitro oocyte maturation in llamas, maturation rates varied from 30.4% to 81% (Del Campo et al. 1994, 1995_a, Ratto et al. 2005, Sansinena et al. 2003, 2007, Trassoras 2009). The rates obtained in these studies using llamas are relatively greater than the overall maturation rate of 20.8% achieved in the present study with alpacas. However, in the present study the 48.5% maturation rate of MNO collected by aspiration and incubated in media supplemented with FBS is comparable to that of previous reports in llamas.

Comparing the maturation rates in the aforementioned studies with those obtained in the present study is difficult as these studies were conducted in llamas and employed various incubation times (28 to 36 hours), collection techniques (aspiration, slicing, aspiration plus slicing), media (chemically defined, serum supplemented), and different environmental conditions (the presence or not of somatic cell co-culture) (Del Campo et al. 1994, 1995_a, Ratto et al. 2005, Sansinena et al. 2003, 2007, Trassoras 2009). In regard to incubation times reported in these previous studies, the incubation time utilized in the present study is shorter than that of the above-mentioned studies. However, it has been shown that incubation of SAC oocytes longer than 28 hours elicit no significant difference over the rate of oocytes that reach maturation (Ratto et al. 2005).

The results in the present study reinforce the benefits of serum (FBS) addition to in vitro media. The composition of the media used for in vitro oocyte maturation plays an integral role in the developmental competence of the oocyte. The benefit of serum is still not completely understood, as the composition of serum remains largely undefined. However, certain components of serum that benefit in vitro culture for oocyte maturation and embryo production have been identified including proteins, hormones, and growth factors (Holzer et al. 2007). Although the present study utilized a chemically defined media containing hormones, proteins, and 1 growth factor (EGF), all MNO collected from antral follicles ≥ 2 mm by aspiration had the greatest maturation rates when incubated in media supplemented with FBS than without it. As FBS also contains hormones, growth factors and other unknown components, it is likely that the addition of FBS enhanced in vitro culture conditions for oocyte maturation. In humans, the addition

of serum to in vitro culture media proves beneficial by increasing oocyte competence compared to oocytes incubated without serum (Hirao 2011). In cattle, serum supplemented media has been shown to increase blastocyst yield on day 6 of in vitro embryo culture (Rizos et al. 2003). In Old World camelids, the only live offspring from in vitro produced embryos were created from oocytes incubated in media supplemented with 10% fetal calf serum (Khatir and Anouassi 2005). Interestingly, supplementation with FBS had no effect on the maturation rate of MNO collected from young alpacas from follicles ≥ 2 mm by aspiration. This could be attributed to the fact that oocytes from young animals are less likely to have undergone morphological, cellular, biochemical, molecular, and epigenetic changes as seen in aged oocytes (Jiao et al. 2012, Jiao and Woodruff 2012). These changes have been shown to impact oocyte maturation, fertilization and potential for embryo production in mice (Wortzman and Evans 2004). Therefore, oocytes recovered from antral follicles ≥ 2 mm of young animals might have an increased potential for developmental competence and no improvements in maturation rate can be made by supplementing media with FBS.

In contrast, the present study demonstrated that MNO from old alpacas collected from follicles ≥ 2 mm by aspiration and incubated in media supplemented with FBS achieved the greatest maturation rates. It has been demonstrated that aged oocytes undergo a variety of detrimental functional changes (Jiao et al. 2012). These changes include structural alterations of the microvilli of the plasma membrane, a decrease in membrane potential which may result in mitochondrial swelling, ZP hardening, premature chromosome separation, and PB degeneration and deviation from the meiotic

spindle (Longo 1981, Miao et al. 2009, Webb et al. 1986). We speculate that oocytes from old alpacas may have benefited from the enhanced components provided by serum including proteins, hormones, and growth factors due to the morphological, cellular, and biochemical changes present in aged oocytes (Jiao et al. 2012, Holzer et al. 2007). Unfortunately, lot-to-lot and batch variations, various contaminants and biosecurity are a concern whenever serum is utilized in a culture system (Hasler 2010, Miao et al. 2009). Therefore, further investigation is warranted to examine the use of a recombinant source such as recombinant serum albumin or a completely defined and serum free source such as a synthetic serum replacement (Desai et al. 1997, Mingoti et al. 2011, Swain and Pool 2012). In cattle and humans, the use of synthetic serum substitute resulted in comparable oocyte maturation and embryo developmental rates to that of serum supplemented media (Desai et al. 1997, Mingoti et al. 2011).

In conclusion, aspiration of oocytes from antral follicles ≥ 2 mm is the most efficient method for oocyte collection (number of MNO/total oocytes collected) and in vitro maturation (number of oocytes reaching maturation/total number of oocytes incubated) in alpacas. The addition of FBS to the in vitro media benefits the maturation rate of oocytes collected from antral follicles ≥ 2 mm compared with those collected from follicles ≤ 1 mm, especially when collected from old alpacas.

Conclusions and implications for future research

Should in vitro technologies become a viable option for production of offspring in SAC, it is worthwhile to note that harvesting oocytes from follicles of various sizes by ovarian slicing may be an important option to consider clinically in the death of animals

of superior genetic merit. Utilizing this technique should increase the overall number of oocytes recovered when compared with aspiration alone. Although slicing recovered a lower proportion of MNO, some of these MNO still reached nuclear maturation as shown in the present study. Thus, for valuable deceased animals, attempts to maximize oocyte recovery and maturation by ovarian slicing may lead to increased embryo yield.

Future studies involving ART in SAC should focus on developing standardized in vitro protocols for oocyte maturation and embryo culture. In particular, alternative options to FBS supplementation may be beneficial to optimize oocyte maturation and potential embryo production. These advances should contribute to the successful production of the first SAC offspring derived from in vitro technologies. To date this has yet to be achieved.

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Appendix A: Composition of Chemically Defined Media

Table 4: Chemically defined oocyte handling media

[illegible]

Table 5: Chemically defined media for in vitro oocyte maturation

[illegible]

Hormone stock preparations for addition to oocyte in vitro maturation media

Epidermal growth factor stock

- 1) Supplies:

Component	Sigma #	Amount
Acetic acid glacial (AAG)	A-6238	100 mL
Epidermal Growth Factor (EGF)	E-9644	0.2 mg

- 2) Make 100 mL of a 10 mM AAG solution: add 57.2 μ L of AAG then add water up to 100 mL
- 3) Add 4 mL of 10 mM AAG solution to the 0.2 mg bottle of EGF (this gives a stock concentration of μ g/mL)
- 4) Aliquot the stock in 26 μ L using 0.5 mL eppendorf tubes and freeze at -80°C

Estradiol 17 β stock

- 1) Supplies

Formula/Component	Sigma #	Amount
Estradiol 17 β	E-2257	1 mg bottle
Ethanol 95%	493511	1000 mL
Holding medium (H-199)		9.8 mL

- 2) Dilute the estradiol in the bottle, with 200 μ L of 95% ethanol (make sure that all the powder dissolves in the ethanol)
- 3) Add 9.8 mL of the holding medium to the bottle (gives a stock concentration of 100 μ g/mL)
- 4) Aliquot 0.26 mL of the stock in 1.5 mL vials and freeze at -80°C

Luteinizing hormone stock

- 1) Supplies

Component	Amount
USDA-LH-B-5	5 mg bottle
Phosphate buffered saline (PBS)	5 mL

- 2) Add 5 mL PBS to bottle of LH (make sure that all the powder dissolves)
- 3) Aliquot the stock in 26 μ L using 0.5 mL eppendorf tubes and freeze at -80°C

Follicle stimulating hormone

- 1) Supplies

Component	Amount
NIDDK-oFSH-20	bottle
Phosphate buffered saline (PBS)	5 mL

- 2) Each bottle will have a slightly different amount of FSH powder
- 3) You will have to calculate for each batch made up; e.g. mg x 1000 = μ g; μ g x 1000 = ng
amount in ng $\div$ 15 = # mL to add to get 15 ng/ μ L stock
- 4) Aliquot the stock in 26 μ L using 0.5 mL eppendorf tubes and freeze at -80°C

Table 6: Stock solutions used to prepare oocyte handling and in vitro maturation media

STOCK	Formula/ Component	Sigma #	Molecular Weight	mM or % (w/v)	Quantity (g)	H2O mL (up to)	0.9% NaCl (up to)	Phenol red (g)	Filter (Y/N)	Storage time	Storage temp	How to store
ALA-GLU	Alanyl-Glutamine	A-8185	217.2	100	0.434	20			Y	3 months	4°C	50 mL tube
B	NaHCO ₃	S-5761	84.01	250	2.100	100		0.005	Y	1 month	4°C	50 mL tube
BSA	Bov Ser Albumin	A-6003	~55,000	10%	2.000	20			Y	2 weeks	4°C	50 mL tube
C	CaCl ₂ · H ₂ O	C-3881	147	200	0.588	20			Y	3 months	4°C	50 mL tube
CAFN	Caffeine	C-0750	194.2	50	0.194	20			Y	1 month	4°C	50 mL tube
CYSTMN	Cysteamine	M-6500	113.6	10	0.011		10 mL		N	3 months	-20°C	0.5 mL vial
CDM:	NaCl	S-9625	58.44	710	8.298							
	KCl	P-5405	74.55	60	0.895							
	KH ₂ PO ₄	P-5379	136.1	10	0.272							
	Na-Citrate · 2H ₂ O	C-5920	294.1	5	0.294							
	TOTAL:			785		200			Y	3 months	4°C	50 mL tube
EAA'S	BME AA sol 50x	B-6766		73.56						1 year	4°C	100 mL bottle
EDTA	C ₁₀ H ₁₆ N ₂ O ₆	E-6635	292.2	1	0.037	100			Y	3 months	4°C	50 mL tube
F	D-Fructose	F-3510	180.2	200	0.721	20			Y	1 month	4°C	50 mL tube
G	D-Glucose	G-6152	180.2	350	1.261	20			Y	1 month	4°C	50 mL tube
Gentamicin	Gentamicin	G-1272	Buy from Sigma						N		4°C	
Glycn	Glycine	G-7126	75.07	490	0.736	20			Y	1 month	4°C	50 mL tube
H-1:1	HEPES free acid	H-4034	238.3	100	2.383							
	HEPES Na salt	H-3784	260.3	100	2.603							
	TOTAL:			200		100		0.005	Y	1 month	4°C	50 mL tube

Continued

Table 6 continued

HP-Mat	Heparin Na salt	H-3393	5000	1%	0.020		2 mL		N	1 month	4°C	50 mL tube
HP-Fert	Heparin Na salt	H-3393	5000	0.10%	0.020		20 mL		N	2 months	-20°C	0.5 mL vial
MI	Myo-Inositol	I-7508	0.998	277	0.998	20			Y	1 month	4°C	50 mL tube
L-L	Na L-lactate	L-7022	1.121	1000	1.121	10			Y	1 month	4°C	50 mL tube
MS	MgSO ₄	M-2643	0.12	50	0.120	20			Y	3 months	4°C	50 mL tube
NaCl	NaCl	S-9625	5.844	5000	5.844	20			Y	3 months	4°C	50 mL tube
NEAA'S	MEM AA sol 100x	M-7145		67.04						1 year	4°C	100 mL bottle
P	Na-Pyruvate	P-4562	0.11	50	0.110	20			Y	2 weeks	4°C	50 mL tube
PH	Penicillamine	P-4875	0.037	2.5	0.037							
	Hypotaurine	H-1384	0.011	1	0.011							
	TOTAL:			3.5		100			Y	3 months	-20°C	1 mL vial
T	Taurine	T-8691	0.025	10	0.025	20			Y	3 months	-20°C	1 mL vial