METABOLIC ANALYSIS OF GLUCOSE, PYRUVATE, AND GLUTAMINE IN DOG OOCYTES COLLECTED FROM DIFFERENT SIZED FOLLICLES AND MATURED IN VITRO

by

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Abstract

Current in vitro maturation (IVM) systems for domestic dog oocytes are inefficient, largely due to the species' unique reproductive physiology. The size of donor follicle influences developmental competence of dog ovarian oocytes. Specifically, oocytes from follicles > 2 mm in diameter complete in vitro nuclear maturation at a higher rate than those from smaller follicles. The objective was to determine the influences of follicular size, maturation time, and meiotic status on oocyte metabolism. We hypothesized that metabolic patterns differed between oocytes from small versus large follicles. Oocytes (n = 531) from adult ovaries were collected and grouped based on follicular size (small, < 1 mm, n = 252; medium, 1 to 2 mm, n = 231; and large, > 2 mm, n = 48). Oocytes were cultured for 0, 24, or 48 hours at 38.5˚C in 5% CO_2 in 80 µL of TCM 199 + 25µM β-mercaptoethanol + 10 ng/ml epidermal growth factor + 0.25 mM pyruvate + 2.0 mM glutamine + 0.1% polyvinyl alcohol + 0.03 mg/ml streptomycin + 0.03 mg/ml penicillin G sodium (IVM medium), assessed for metabolism and evaluated for nuclear status. For metabolic assessments, oocytes were incubated for 3 h in 3 µl of IVM medium containing (1) 0.005 mM [0.064 µCi/µl] D-5^{3}H-glucose (glycolysis) + 1 mM D-6^{14}C [0.053 µCi/µl] glucose (glucose oxidation) or (2) 0.001 mM [0.041 µCi/µl] L-G-^{3}H-glutamine + 1 mM [0.027 µCi/µl] 1-^{14}C pyruvate, placed on the lid of a centrifuge tube containing 25 mM $NaHCO_3$ and trapped radioactivity was measured using a β-counter. Only oocytes at an appropriate meiotic stage for each culture period (n = 380) were included in data analysis (e.g., germinal vesicle stage at 24 and 48 h culture were excluded). Differences in metabolism among groups were analyzed by ANOVA (main effects being follicular class, culture interval, and meiotic status). Oocytes recovered from large follicles metabolized significantly more pyruvate, glutamine, and glucose (via glycolysis) than those from small ones ($p < 0.05$). Across meiotic stages and follicular sizes, glycolytic rate was lowest in oocytes cultured for 24 hours ($p <$

0.05) compared to 0 or 48 hours. Metaphase II oocytes had a significantly higher glycolytic rate than those at other meiotic stages ($p < 0.05$). At culture onset (0 h), oocytes from small follicles predominately used pyruvate ($p < 0.05$), while oocytes from larger follicles ($p < 0.05$) predominately metabolized glucose. The present data suggests that dog oocytes preferentially use glucose as an energy substrate and that increasing glycolytic rate correlates with meiotic maturation. In addition, oocytes collected from large follicles exhibit increased metabolic capabilities that may be responsible for their increased developmental competence during IVM.

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CHAPTER 1 - LITERATURE REVIEW

Introduction

The field of assisted reproductive technology is constantly expanding. Its applications are now commonplace in fields ranging from human infertility treatment to livestock production. Techniques such as in vitro fertilization, artificial insemination, gamete cryopreservation, and embryo transfer are all examples of what science can do to enhance natural reproductive efforts.[1] The application of such technology to carnivores has progressed at a much slower pace than it has in other species. This is especially true with respect to the canids. Interestingly, the reproductive physiology of the domestic dog and its wild counterparts differs in many respects from the norms established for most other mammals. These differences have slowed down technological advances in canine reproduction but have not stopped them. The following literature review will explain the need for further research in the field of canid reproduction. It will also cover the basic reproductive physiology of the female dog, the successes up to this point in canine assisted reproduction, the current state of in vitro maturation of canine oocytes, and finally the role of energy substrate supplementation in oocyte maturation in vitro.

Canid Research

The need for further research in the domestic dog is multifaceted. One of the most popular applications of canine research to date has been the use of dogs as a research model for human disease.[2-4] With the abundance of genetic disorders in the domestic dog, many of which bear striking similarities to the correlating conditions in humans, there is a need to maintain highly specific genetic lines to facilitate research in this field. However, because the individuals in these canine colonies need to be bred with specific genotypes, offspring production generally is not simple. Specifically, perpetuating a genetic disorder

mandates the mating of close relatives by inbreeding or line breeding that has been shown to reduce sperm quality, fertility, and fecundity.[5] An array of assisted breeding tools, including oocyte in vitro maturation (IVM), in vitro fertilization (IVF), and embryo transfer would be particularly useful for managing these specialized colonies. These technologies also may permit the rescue of genetic material from individuals that fail to reproduce, are genetically under-represented in the population, geriatric, terminally ill, or recently deceased.

Unfortunately, reproductive knowledge pertaining to the domestic dog is incredibly sparse when compared to other domestic species. This is because of a lack of research effort in this discipline, combined with the inherent physiological differences that characterize the canine species (discussed in the following section). Further research focused on canine reproduction, specifically on the oocyte, will help facilitate these invaluable human disease studies.

In addition to helping manage domestic dog colonies used for studying human diseases, more extensive canine reproductive research may be able to help save the wild counterparts of the domestic dog. Of the 36 species in the family Canidae, nine are currently considered threatened or endangered.[6] For many of these species, survival may depend on the application of assisted reproductive technology to maintain genetic diversity of small, fragmented populations. This will only become a possibility once these techniques have been perfected in the domestic dog.

Reproduction in the Bitch

When compared to other domestic species, the reproductive physiology of the dog is unique in many aspects. Dogs are considered a monoestrous species, meaning they have only one or two periods of sexual receptivity each year during which they ovulate.[7] Unlike most other mammals where ovulation occurs once oocytes have reached metaphase II, canids ovulate an oocyte that is immature and incapable of fertilization.[8] In the domestic dog, ovulation regularly occurs two days after a sharp peak in luteinizing hormone (LH) levels.[9]

Once in the oviduct, the oocyte must undergo a second meiotic division to complete maturation. This has usually taken place 48 to 72 hours post-ovulation, marking the point at which fertilization can occur.[7,10,11] Fertilization remains possible for approximately 48 additional hours.[10]

Another unique characteristic of canine reproduction is the striking similarity in hormonal profiles between pregnant and non-pregnant females. The length of the luteal phase in non-pregnant females correlates closely to the gestation length of a pregnant female (approximately 9 weeks). Hormonal differences between these two states are usually only recognizable around the time of parturition when pregnant females experience increasing estrogen levels not seen in non-pregnant females.[7,8] Since most non-pregnant dogs experience some level of mammary gland enlargement during their luteal phase, some refer to this period as a physiological pseudopregnancy.[8]

An additional distinctive quality of the bitch that tends to complicate reproductive research is the unreliable timing of the estrus cycle. Observation of standing heat, or in other words, a female willing to stand for copulation, is a reliable indicator that a bitch is in estrus. Unfortunately, the onset of estrus and the time of ovulation are not directly related, and can actually be highly variable among different bitches. In fact, the timing and duration of all stages of the reproductive cycle seem to vary considerably among individual bitches.[7]

The four stages of the reproductive cycle of the bitch are proestrus, estrus, diestrus (also known as metestrus), and anestrus. Compared to other species, both proestrus and estrus are lengthened.[7] During proestrus estrogen levels are on the rise and serosanguinous vaginal discharge and vulval swelling may be noted. Male dogs are beginning to show interest during this time but are rejected by the female. The transition into estrus is marked by decreasing estrogen levels and increasing progesterone levels. On average, bitches stay in estrus for 9 days, however the marked variability among individuals accounts for an accepted range from 3 days to several weeks.[8] Toward the end of estrous stage, the female begins to reject males. This stage is followed by diestrus, a period during which circulating progesterone remains elevated. If a bitch is pregnant, the

entirety of her pregnancy and lactation are considered to take place during diestrus. If non-pregnant, the duration of diestrus is slightly harder to define but accounts for the completion of the luteal phase. The final stage of the reproductive cycle is anestrus. This stage lasts an average of 4 months, during which time the ovary becomes inactive. It has been demonstrated that increasing levels of FSH at the end of this stage stimulate the return of ovarian activity and follicular development and bring anestrus to an end, thus beginning the next reproductive cycle.[12]

Successful Advances in Canine Assisted Reproductive Techniques

Although the field of canine assisted reproduction has been slow to progress overall, certain techniques are already achieving success. For instance, semen cryopreservation, artificial insemination and embryo transfer using in vivo derived oocytes are all examples of techniques that have made great strides so far.

The first of these techniques, canine semen cryopreservation, is a constantly evolving field of research. Because of the relatively small ejaculate volume in the dog, comparing different cryopreservation protocols on a single ejaculate has been a challenging obstacle.[13] As a result of this, development of freezing protocols and extenders for cryopreservation use in the dog have been largely based on those already in place for other species.[14] While no identical freezing or thawing method seems to work for all dogs, empirically derived protocols seem to accommodate most samples, and have allowed cryopreservation of canine semen to become commonplace.[13]

Another success in canine assisted reproduction has been artificial insemination. There are several artificial insemination techniques in use, including intravaginal insemination, transcervical insemination, and surgical insemination. Intravaginal insemination is often used with fresh semen; however, if frozen semen is to be used, an intrauterine insemination (IUI) technique such

as transcervical insemination or surgical insemination should be used. Because the thawing process can compromise the quality of the sample, it is better to bypass the cervix altogether to increase the odds of success.[15-18]

Transcervical inseminations have traditionally been more difficult due to the anatomy of the canine reproductive tract. Some of the complicating factors include a comparatively longer vagina than other species and a prominent dorsal median fold in the vagina that creates a misleading pseudocervix.[19] The dorsal median fold also impinges on the size of the vaginal lumen, limiting the size of equipment that can be passed through the paracervical area.[20]

Despite the anatomical challenges, two transcervical insemination techniques have been developed and are currently in use. The first technique (also known as the "Norwegian" method)[15] has achieved conception rates using frozen-thawed semen between 41.5 and 73.6 %[17,21 22 23]. In a recent retrospective study by Thomassen, et.al., the utilization of the Norwegian method to artificially inseminate frozen-thawed semen into 526 bitches resulted in an overall whelping rate of 73.1 percent and a mean litter size of 5.7 pups.[24]

The second method of transcervical insemination uses endoscopic visualization to pass a plastic catheter through the cervix. With a single, timed, endoscopic insemination of frozen-thawed semen into 137 Greyhound bitches, a pregnancy rate of 89.4% and a mean litter size of 6.9 pups was achieved by Pretzer, et.al..[25] Previous studies using endoscopic inseminations with frozen-thawed semen achieved a whelping rate of 57.9%[18] and a conception rate of 80%[16].

Finally, embryo transfer with in vivo derived oocytes has also begun to achieve reasonable levels of success. Embryo transfer using embryos at the 8-cell stage through the blastocyst stage have all resulted in pregnancy in the dog.[26] In the silver fox (*Vulpes vulpes*), a recipient female gave birth after embryo transfer of four expanded blastocysts and one 16-cell stage embryo.[27] More recently,early stage embryos (zygote to 4-cell embryos) collected after excision and flushing of both donor uterine tubes and transferred to the recipient's uterine horn resulted in pregnancy in one of eight bitches in the

study.[28] Even though advances are being made, embryo transfer in canine species has not been easily reproducible. In order to use embryo transfer commercially in this species, a reliable method for induction of ovulation and estrus synchronization must be established.

In Vitro Maturation (IVM)

In vitro maturation (IVM) of canine oocytes has proven to be significantly more challenging than it has been in other domestic or laboratory species. As previously described, bitches ovulate an immature oocyte that requires approximately 48 hours in the oviduct to complete maturation in vivo. This means that an equivalent amount of time is necessary to mature canine oocytes in vitro to the metaphase II stage. Comparatively, this takes 10-13 hours in mouse oocytes[29] and 16-24 hours in cow ova.[30] Another characteristic that is unique to canine oocytes is their exposure to high levels of progesterone in the follicular environment instead of the increased estrogen levels that dominate other mammalian species.[31] These basic differences mean that culture and maturation of canine oocytes may need vastly different in vitro conditions than those previously established for other species. This is evident when examining the success rates of studies on canine IVM to date. Current protocols that have been derived based on those developed for other species have yielded poor results, with rates of maturation to the metaphase I or II stages averaging approximately 20%.[32,33],[34-44] Comparatively, feline IVM to the metaphase II stage averages 40-60% after 24 hours of IVM[45] and cattle IVM to the metaphase II stage averages 80-90%.[46,47]

Oocyte collection

The simplest way to gain access to dog oocytes is to collect ovaries after a standard ovariectomy or ovariohysterectomy is performed. After ovaries are obtained, it is common to isolate oocytes by slicing the cortex lengthwise and crosswise with a scalpel blade, followed by rinsing with a culture medium. This technique consistently produces higher recovery rates than follicular aspiration,

which is common in many mammalian species.[31] Poor success of follicular aspiration is due to the relatively short window of time that follicles are visible on the surface of the ovarian cortex and also to the small dimensions of the canine ovary.[31] Additionally, the ovarian bursa that surrounds the canine ovary makes the use of a transabdominal laparoscopic recovery technique very difficult.[1]

After oocyte recovery is complete, selection of oocytes most likely to mature successfully is the next important step. Several morphological criteria such as diameter, cumulus conformation and morphology are generally used to select oocytes for IVM.[31] It has been shown that oocytes greater than 100 μm in diameter develop to MI/AI/MII at a higher rate (20%) than do oocytes less than 100 μm in diameter (4%).[48] Additional research has demonstrated that only oocytes with two or more layers of intact cumulus cells should be selected for IVM/IVF, as those with fewer layers did not extrude the first polar body and showed signs of degeneration by 48 hours of culture.[36]

Culture methods and media

Once oocytes are collected, the most common culture method used for maturation involves drops of medium covered by mineral oil.[31] Culture on a bovine cumulus cell monolayer has also been reported; however, no significant effect on maturation was observed.[35] An investigation of co-culture with canine oviductal epithelial cells showed improved oocyte maturation rates, however this was only noted after 96 hours of culture.[49] Furthermore, the use of an oviduct that was isolated to serve as an in vitro culture environment was shown to have a positive effect on nuclear maturation and oocyte survival, indicating that in addition to the oviductal cells themselves, an intact oviduct may provide unique growth factors or secretory products that play a role in oocyte maturation.[50]

Both simple and complex media have been used for culturing domestic dog oocytes. The most common simple varieties being used are Modified Krebs Ringer Bicarbonate and Synthetic Oviductal Fluid (SOF), and the most common complex media being used is TCM 199.[31] It has been shown that oocytes

cultured in TCM 199 completed nuclear maturation at a higher rate than those cultured in another complex media, CMRL 1066.[41] In addition, TCM 199 was also shown to produce increased proportion of oocytes developing to the MI/AI/MII stage than the simple media, SOF.[51]

Impact of donor reproductive status, follicle size and age

Because of the random acquisition of ovaries from donors of various reproductive statuses typical of most IVM studies, many have questioned whether the stage of the reproductive cycle plays a role in the ability of collected oocytes to mature in vitro. After a retrospective analysis of data from 1,643 oocytes collected from bitches of known reproductive stage, it appears that the stage of reproduction has no influence on the subsequent ability of oocytes to mature successfully in vitro.[52] It was also determined that the season of year in which oocytes are collected plays no role in their ability to mature in vitro either.[52] This data stands in opposition to that presented by Luvoni, who indicated that cumulus-oocyte complexes (COCs) collected and matured from bitches in late pro-estrous retained open communication between the oocyte and its cumulus cells via gap junctions.[37] This communication was closed in COCs collected from bitches in anestrous. Because the open communication allowed by gap junctions was correlated to an increased ability to resume meiosis and reach nuclear maturation, this data indicated that oocytes collected from bitches in anestrous were unsuitable for in vitro maturation.[37]

One factor that was determined to have a substantial effect on in vitro oocyte maturation was the size of the donor follicle the oocyte was recovered from. It has been demonstrated that oocytes collected from follicles greater than 2 millimeters in diameter had an almost 80% rate of reaching meiotic competence, compared to 16.9 % in follicles less than 0.5 millimeters, 26.1% in follicles from 0.5 to 1 millimeter, and 38.4% in follicles from 1 to 2 millimeters. [52] The relationship between increasing antral follicle size and increased

developmental capacity of oocytes has been demonstrated in the bovine as well.[53]

The effect of donor age on oocyte quality has also been investigated. One study found that oocytes collected from bitches between 1 and 6 years of age had greater potential to mature than those collected from bitches 7 years of age and older.[48] These findings were corroborated by another study that showed that adult bitches less than seven years of age were more likely to provide both higher quality and increased quantity of oocytes for in vitro study than very old or very young animals.[54]

Additional culture supplements

Other culture supplements that have been studied include substances thought to prevent oxidative damage like β-mercaptoethanol and insulin-transferrin-selenium. Both substances seem promising as research has indicated a beneficial effect of supplementing culture media with 50 or 100 μM β-mercaptoethanol,[42] as well as increased maturation rates that accompanied supplementation of TCM 199 with insulin-transferrin-selenium.[51] Another supplement that has received research attention is epidermal growth factor (EGF). Addition of EGF to culture media is thought to trigger oocyte nuclear maturation, cumulus expansion, and promote developmental competence via cell signaling pathways in the cumulus cells.[55] It was shown that exposure to 20 ng/ml of EGF improved IVM of canine oocytes to the MII stage.[42] In addition, it appears that EGF in conjunction with FSH and LH enhanced granulosa-cumulus expansion of cultured canine oocytes, however it did not significantly effect the percent of oocytes maturing to the MI/MII stage.[56]

Protein supplementation in the form of serum or bovine serum albumin has been described in several canine IVM media methods.[34,35,43,44,57] This type of protein supplementation has been correlated with high percentages of canine oocytes with unidentified nuclear material after fixation and assessment of meiotic maturation.[57] It has since been determined that protein-free media can be

used to mature canine oocytes in vitro, making it possible to eliminate any potential negative impacts of serum or BSA in culture media as well as eliminating the effect of unknown substances or growth factors that are frequently contained in commercial serum or bovine serum albumin.[41]

Supplementation of various hormones has also been investigated. The addition of LH and FSH does not appear to enhance maturation of canine oocytes in vitro.[58] In fact, culture media supplemented with luteinizing hormone seems to be detrimental to nuclear maturation.[41] Short exposure to equine chorionic gonadotropin, on the other hand, has been shown to improve canine oocyte IVM by promoting resumption of meiosis and enhancing developmental capacity.[40] Supplementation with estradiol-17β alone, progesterone alone, or a combination of the two hormones all increased the proportion of oocytes maturing to the MII phase; however, this was only noted in oocytes recovered from donors in the follicular phase of their cycle.[59] As mentioned previously, canine oocytes are exposed to a comparatively unique hormonal profile as they complete maturation in the oviduct. It may therefore be necessary to provide supplementation more tailored to the natural in vivo hormonal environment in order to achieve higher levels of IVM success.[36]

Influence of sperm penetration

Another factor that may play a role in the maturation of canine oocytes is penetration by spermatozoa. The ovulation of immature oocytes in canid species means that interaction with spermatozoa before maturation has occurred is highly likely. This is especially true considering the lengthy amount of time that canine sperm can survive within the female reproductive tract. Large numbers of spermatozoa remain motile within the uterus for 4-6 days after mating, with smaller numbers of spermatozoa still present up to 268 hours after mating.[60] In fact, spermatozoa even seem to be stored within the uterine glands of the bitch.[60] Even if insemination is not well correlated to the time of ovulation it is likely that there will still be spermatozoa ready when timing for fertilization is correct. These

observations lead some to investigate whether or not sperm penetration had any effect on the resumption of meiosis in immature oocytes. Data indicated that indeed, sperm penetration did play a role in oocyte maturation, with results indicating a higher percentage of fertilized oocytes reaching the metaphase I or II stage than control (unfertilized) oocytes, and a lower percentage of fertilized oocytes remaining at the germinal vesicle stage than control.[38] Subsequent research has challenged the validity of those findings, documenting only three out of 112 immature oocytes that were penetrated by spermatozoa after ovulation and artificial insemination.[61] It has been suggested that penetration of immature oocytes by spermatozoa in vitro may be an artifactual finding induced by suboptimal culture conditions.[62]

IVM Conclusions

Despite the strides that have been made to this point, IVM in canid species is in need of further research to achieve maturation rates comparable to those already seen in other domestic species. Due to the slow progress in the field of canine IVM, other assisted reproductive techniques such as in vitro fertilization (IVF) and embryo transfer have lagged behind as well. There has yet to be a successful pregnancy (resulting in the birth of live young) from an embryo transfer where the embryo was derived from an oocyte matured and fertilized in vitro. In fact, only a few have reported on IVM/IVF in the dog that led to any embryonic development at all.[34,35,41,63,64]

Oocyte Metabolism

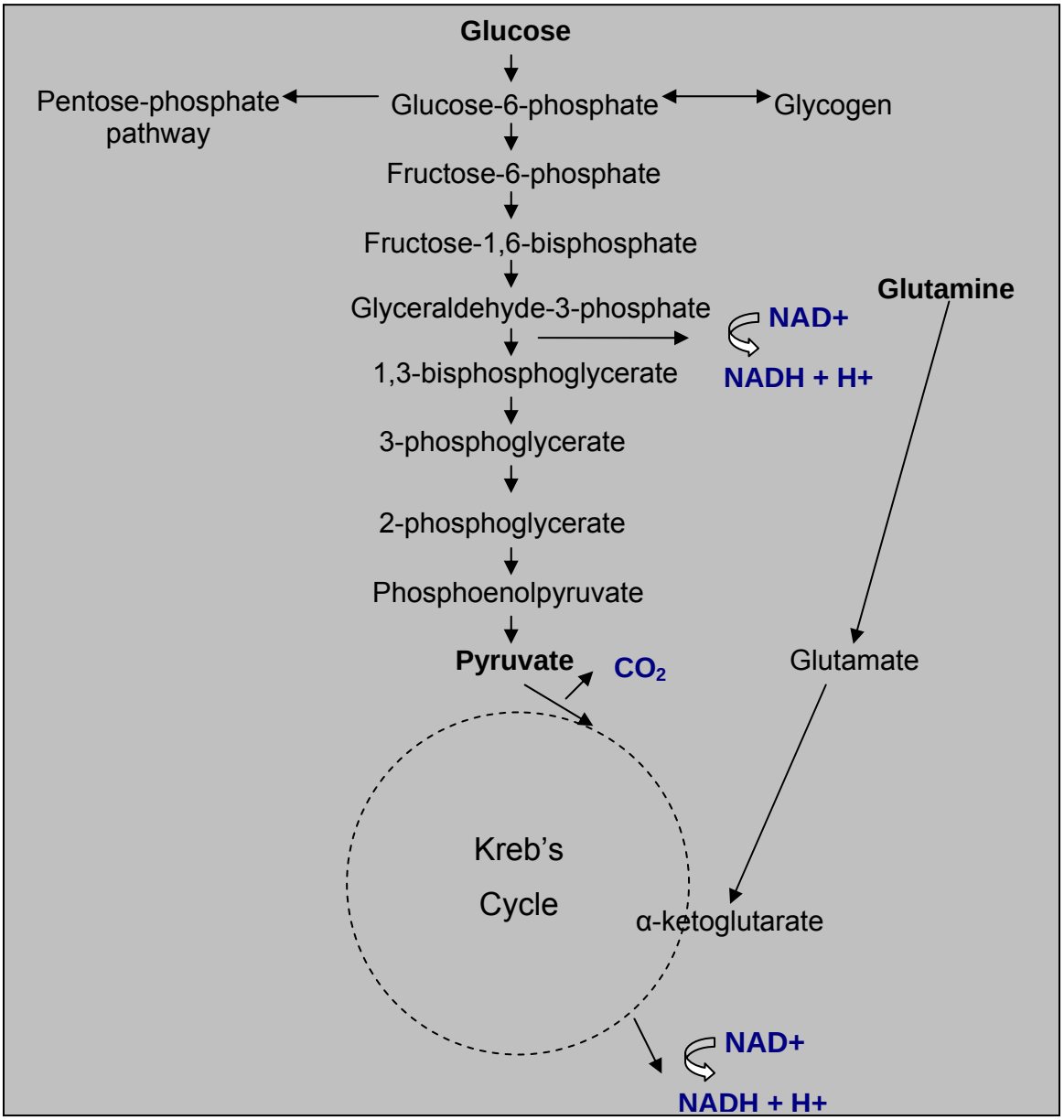
As discussed above, the in vitro maturation system for canine oocytes is not yet up to par with those in place for other domestic species. One component of this system that has yet to be fully elucidated is the proper supplementation of energy substrates into the culture medium. Since the availability of energy substrates is vital to oocyte maturation, providing the necessary substrates at the

ideal concentrations and times may lead to a more reliable canine oocyte maturation system.

One of the most common ways to assess individual oocyte metabolism is via the hanging drop method.[65,66] Using this method, oocytes are incubated in individual 3 µl drops of in vitro maturation medium on the lid of a microcentrifuge tube. The medium contains various radiolabeled substrates such as D-5-^{3}H-glucose, D-6-^{14}C-glucose, etc. The lid of the microcentrifuge tube is then closed over the body of the tube which contains 1.5 ml of 25 mM $NaHCO_3$. The $NaHCO_3$ serves as a trap for $^{14}CO_2$ (released as pyruvate enters the Kreb's cycle) or ^{3}H (released during the reduction of NAD+ to NADH) (Fig 1.1). The amount of $^{14}CO_2$ or $^{3}H_2O$ produced due to individual oocyte metabolism can then be measured by analyzing the radioactivity of the $NaHCO_3$ trap using a β-scintillation counter.

More specifically, $^{3}H_2O$ produced from D-5-^{3}H-glucose is used as a measure of glucose metabolism through the glycolytic (Embden-Meyerhof) pathway.[67] Production of $^{14}CO_2$ from D-6-^{14}C-glucose is used to measure oxidative glucose metabolism via the Kreb's cycle.[67] Similarly, production of $^{14}CO_2$ from 1-^{14}C pyruvate and $^{3}H_2O$ from L-G-^{3}H-glutamine provides a mechanism to measure of the oxidative metabolism of these two substrates.[67] Although the technology is available to carry out studies on oocyte metabolism, up to this point there has been little research focused entirely on oocyte metabolism in the dog. The most extensive metabolic analysis has been done in cattle and mouse oocytes[68-70]. Researchers have investigated which energy substrates are essential for oocyte survival and maturation in these species. It has been shown that denuded mouse oocytes can reach maturation with supplementation of either pyruvate or oxaloacetate alone.[71] Supplementation with only glucose, lactate or phosphoenolpyruvate, however, was not sufficient to allow maturation to occur. For glucose to be the sole energy source for denuded mouse oocytes, follicular cells had to be present in the culture medium.[71] As a result of these findings, it is now generally accepted that mammalian oocytes utilitze glucose via the cumulus cells. [72] These cumulus cells are thought to

Figure 1.1 Simplified diagram of several metabolic pathways (Adapted from Alberts, Bray and Lewis[73]). The H+ and CO_2 molecules highlighted in blue represent the locations where radiolabelled ^{3}H and ^{14}C are released from the pathway and able to be trapped and measured by the hanging drop method.



break glucose down into pyruvate or other intermediates of the Krebs cycle that the oocyte is able to utilize.[72] It follows then that anaerobic glucose metabolism is significantly less important than oxidative metabolism when it comes to energy production in mammalian oocytes.[67] It is important to keep in mind, however, that oocytes maturing in vivo are never without their surrounding cumulus cells. While denuded oocytes (which are commonly used in a research setting) may primarily utilize the Krebs cycle to support their maturation, cumulus-enclosed oocytes are likely able to utilize both glycolysis and the Krebs cycle.[74]

An additional facet of oocyte metabolism that has been investigated is the relationship between metabolic rates and maturational status. Rieger and Loskutoff, 1994, found that pyruvate, glycine and glutamine metabolism in cattle oocytes significantly increases around the time at which the meiotic maturation is completed (i.e, metaphase II stage, approximately 12 to 18 hours after IVM), while glucose metabolism remained low throughout the culture period.[67] They concluded that oxidative metabolism increases as maturation proceeds and is responsible for the bulk of cellular energy production of cattle oocytes in vitro.[67] Similar findings were observed in mouse oocytes by Downs, et al.[74] Their data indicated that oocytes that had resumed meiosis consumed significantly more pyruvate than oocytes arrested at prophase I or metaphase II. Once again, glucose metabolism was low and changed little throughout the culture period.[74]

Although oocyte metabolism in the dog has yet to be studied as intensively as it has in other species, initial research seems to indicate that the pattern of metabolic activity of dog oocytes is distinct from that of other species. Data indicates that glucose seems to be the energy substrate used most extensively by denuded canine oocytes.[75] This is different from other species studied up to this point. It has also been shown however, that culture media supplemented solely with glucose may not be enough to ensure successful oocyte maturation in the dog.[41] Further research indicated that this may be due to the absence of glutamine,[76] however that theory has since been discounted. A subsequent study indicated that the supplementation of glutamine into culture medium had no significant impact on nuclear maturation in the dog oocyte.[77]

Similarly, pyruvate supplementation also seems to have no impact on developmental competency.[77] Other research has indicated that oocytes cultured under high oxygen tension with high glucose concentrations and no pyruvate supplementation are developmentally inhibited.[41] Further investigation is necessary to continue to piece together the intricacies of oocyte metabolism in the canine.

Conclusion

Reproductive work with the canine species has proven to be challenging on many levels. Physiological differences make the canine stand apart from many of its mammalian counterparts. In order for assisted reproduction to continue to advance in this species, the ability to mature oocytes in vitro must be vastly improved. When IVM can reliably produce large numbers of oocytes at the metaphase II stage, it will allow for IVF, embryo transfer, and even intracytoplasmic sperm injection to be applied to the domestic dog. Elucidation of the metabolic needs of the canine oocyte will likely contribute to achievement of a more reliable in vitro oocyte culture system. This is just one piece of the puzzle that must be studied to continue to increase insight into the mysteries of canine reproduction. As the knowledge base surrounding canine reproduction continues to grow, so will the success of assisted reproduction in this species.

CHAPTER 2 - METABOLIC ANALYSIS OF GLUCOSE, PYRUVATE, AND GLUTAMINE IN DOG OOCYTES COLLECTED FROM DIFFERENT SIZED FOLLICLES AND MATURED IN VITRO

Introduction

Current IVM systems for domestic dog oocytes are inefficient, largely due to the species' unique reproductive physiology. The size of donor follicle influences developmental competence of dog ovarian oocytes. Specifically, oocytes from follicles > 2 mm in diameter complete in vitro nuclear maturation at a higher rate than those from smaller follicles. The objective of this study was to determine the influences of follicular size, maturation time, and meiotic status on oocyte metabolism in vitro. We hypothesized that metabolic patterns would differ between oocytes collected from small follicles versus large follicles.

Materials and Methods

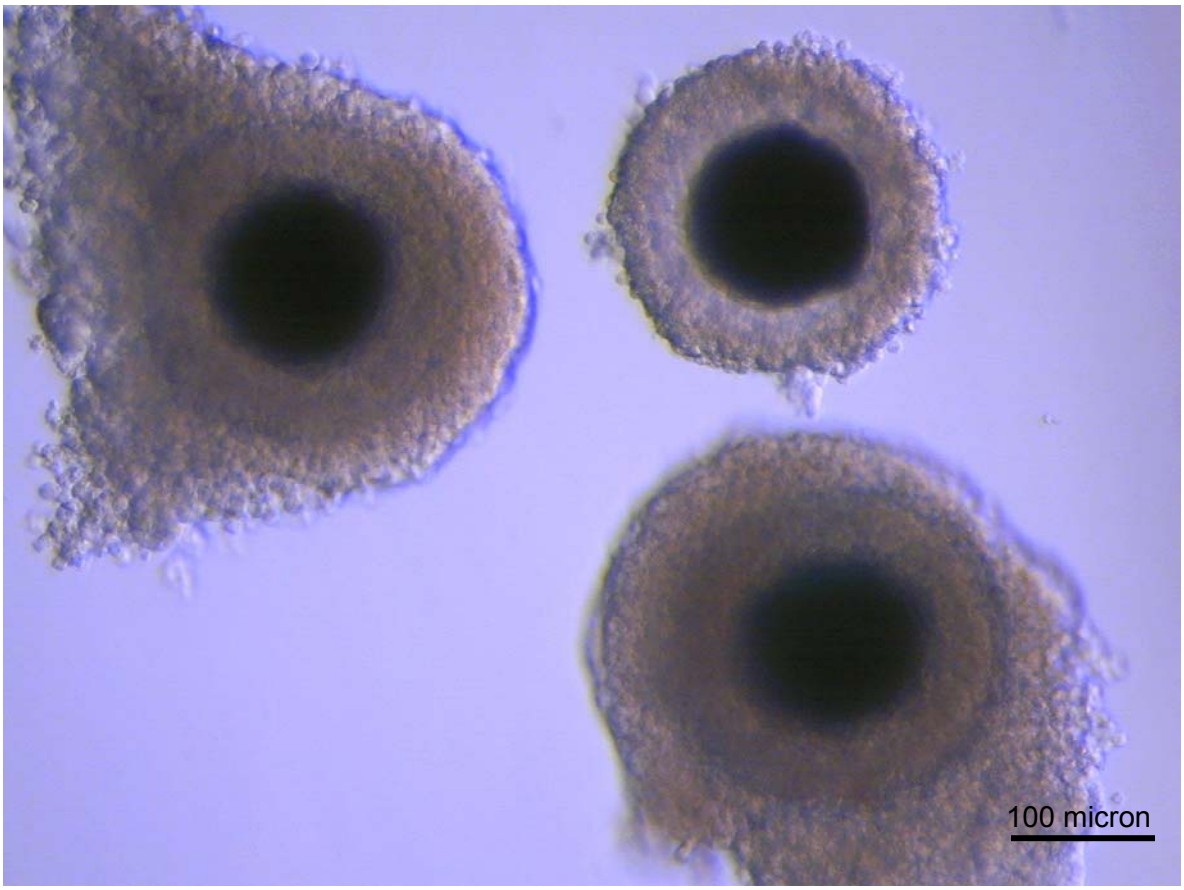
Collection of Canine Oocytes

Oocytes were recovered from ovaries of bitches (n = 97) ranging in age from 6 months to 6 years undergoing ovariohysterectomy at veterinary hospitals in the Northern Virginia area. Ovaries were transported to the laboratory at the Smithsonian's Conservation & Research Center in Front Royal, Virginia at room temperature (22-25˚C) in 0.9% NaCl + 0.06 mg/ml penicillin G sodium + 0.06 mg/ml streptomycin. Oocytes were collected within 6 hours of surgery by horizontally dissecting each ovary (~5 mm thickness) to observe and measure the size of individual follicles using a small ruler. Follicles were divided into three classes based on their size: small, < 1 mm in diameter (n = 252) oocytes; medium, 1 to 2 mm in diameter (n = 231); and large, > 2 mm in diameter (n = 48).

The cumulus-oocyte-complex (COC) from each follicle was recovered by dissecting the follicular wall with a 20-gauge needle (Fig. 2.1), after which it was grouped with other COCs from the same follicular class in TCM 199 with 25mM HEPES. Only oocytes exhibiting homogenous dark cytoplasm with two or more layers of cumulus cells were selected for use in the study (Fig. 2.2)

Figure 2.1 Dissection of follicular wall with 20-gauge needles.

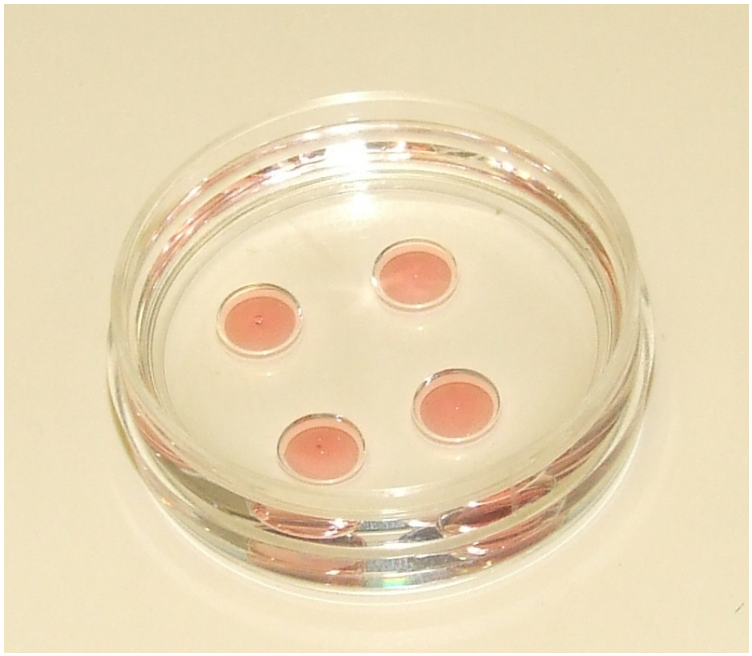
Figure 2.2 Canine oocytes exhibiting homogenous, dark cytoplasm with two or more layers of intact cumulus cells.



Culture

Oocytes were cultured for 0, 24, or 48 hours at 38.5˚C in 5% CO_2 in 80 µL culture drops of TCM 199 + 25µM β-mercaptoethanol + 10 ng/ml epidermal growth factor + 0.25 mM pyruvate + 2.0 mM glutamine + 0.1% polyvinyl alcohol + 0.03 mg/ml streptomycin + 0.03 mg/ml penicillin G sodium (IVM medium) covered by 4.5 ml mineral oil in 35 x 10mm petri dishes (Fig. 2.3). Prior to culture, oocytes were washed twice in IVM medium and incubated for one hour with IVM medium + 5 IU/mL Equine Chorionic Gonadotropin. No more than 9 oocytes were cultured together per 80 µL drop of IVM medium.

Figure 2.3 Culture drops covered with mineral oil.



Metabolic Analysis

After in vitro culture, the cumulus cells were removed by repeatedly pipetting the oocytes with a small diameter (~ 150 micron) glass pipette and the denuded oocytes assessed for metabolic activity using the hanging drop method[66]. Oocytes were incubated for 3 hours (38.5ºC) in individual 3 µl drops of IVM medium containing (1) 0.005 mM [0.064 µCi/µl] D-5^{3}H-glucose + 1 mM [0.053 µCi/µl] D-6^{14}C glucose (+ 1mM pyruvate to adjust the solution to the same pyruvate concentration as treatment 2) or (2) 0.001 mM [0.041 µCi/µl] L-G-^{3}H-glutamine + 1 mM [0.027 µCi/µl] 1-^{14}C pyruvate, placed on the lid of a 1.5 ml microcentrifuge tube. The lid was then closed over the body of the tube which contained 1.5 ml of 25 mM $NaHCO_3$. The $NaHCO_3$ served as a trap for metabolically released $^{14}CO_2$ or $^{3}H_2O$. In addition to the experimental tubes, three control tubes were incubated with 3 µl drops of medium alone to determine the amount of radioisotope that was passively transferred into the $NaHCO_3$ trap (baseline). Three more tubes were incubated with 3 µl drops of medium alone and the medium mixed into the $NaHCO_3$ after the 3 hour incubation period to

determine the total radioactivity (in pmol). Trapped radioactivity was measured by adding 1 ml of $NaHCO_3$ trap solution from each centrifuge tube to 5 ml of scintillation fluid and running each sample through a dual-label channel of a β-counter (Beckman Coulter, Fullerton, CA USA: LS 6500 Multi-Purpose Scintillation Counter). The amount of $^{14}CO_2$ or $^{3}H_2O$ produced due to individual oocyte metabolism was calculated by subtracting the baseline value and dividing by the disintegrations per minute (DPM) value for 1 pmol of each substrate. Using the dual-label setting it was possible to measure ^{14}C and ^{3}H simultaneously, thus D-5^{3}H-glucose and D-6^{14}C glucose (measuring glycolysis and glucose oxidation respectively) were measured together, as were 1-^{14}C pyruvate and ^{3}H-glutamine (measuring pyruvate uptake and glutamine oxidation respectively).

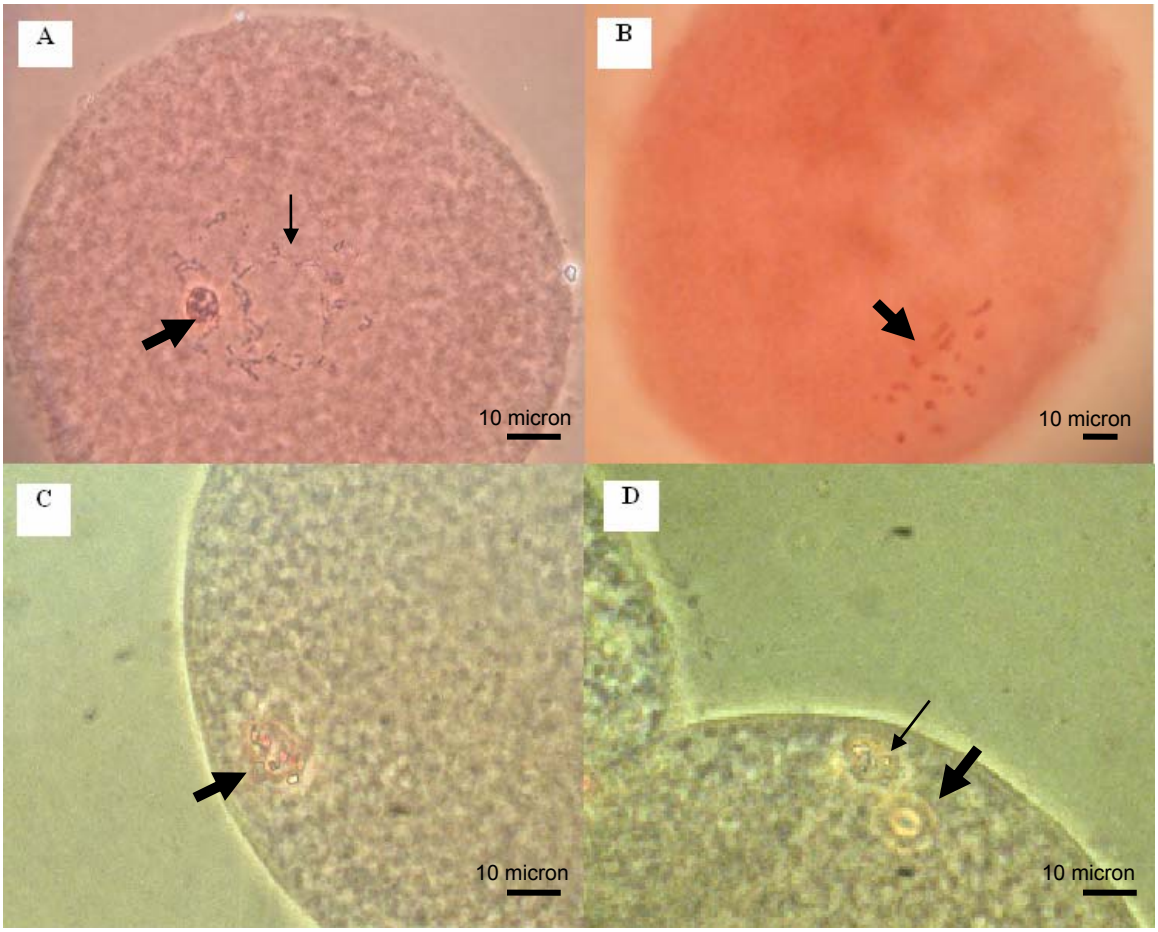
Figure 2.4 Microcentrifuge tubes with oocytes in each lid ready for metabolic analysis via the hanging drop method.

Evaluation of Nuclear Status

After metabolic analysis, oocytes were individually fixed by placing them into wells containing a 1:3 acetic acid:ethanol solution for 48 hours. Each fixed oocyte was then stained using aceto-orcein (1% [w/v] orcein in 45% [v/v] acetic acid), and washed in aceto-glycerol (1:1:3 glycerol:acetic acid:distilled water). Nuclear status was evaluated with light microscopy (400x). Each oocyte was

categorized as being at one of the following stages: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) (Fig. 2.4). Only oocytes at an appropriate stage of nuclear maturation for each culture period (n = 380) were included in data analysis (e.g., oocytes with a germinal vesicle at 24 and 48 hours of culture were excluded).

Figure 2.5 Micrographs of (A) germinal vesicle stage: note prominent nucleolus (thick black arrow) and fine chromatin (thin black arrow); (B) germinal vesicle breakdown stage: chromatin has condensed into clumps (arrow); (C) metaphase I stage: first meiotic spindle is present arrow); and (D) metaphase II stage: second meiotic spindle is present (thin black arrow) as well as the first polar body (thick black arrow). (Micrographs courtesy of Dr. Nucharin Songsasen.)



Statistical Analysis

All data were expressed as mean ± standard error of mean (SEM). Data were transformed using the equation y = log x + 1. Analysis of the transformed data was performed using ANOVA (SigmaStat, SPSS, Inc., Chicago, IL). Due to the non-normality of the majority of the data, Kruskal-Wallis non-parametric one way ANOVA was used, followed by Dunn's multiple comparison. The level of significance was set at 95%.

Results

Maturation Kinetics

During the course of the study 659 oocytes were analyzed for metabolic activity and nuclear status. Of these oocytes, only 379 (57.5%) reached an appropriate nuclear stage for the length of time they were cultured (e.g., GV is only appropriate at 0 hours of culture, GVBD is only appropriate at 24 hours of culture or less, and MI/MII should have been achieved by 48 hours of culture). Across all culture intervals, a total of 170 oocytes reached maturity (defined in this study as MI or MII). This equates to 25.8% of all oocytes in the study completing nuclear maturation. Of the 296 oocytes cultured for 48 hours, 76 reached MI (25.6%) and 45 (15.2%) reached MII.

Immediately after release from the follicle the majority of oocytes were at the GV stage (63.0% ± 6.8) (Fig. 2.6). There were significantly more oocytes at the GV stage at this time point than there were at either 24 or 48 hours of culture ($P < 0.05$) The remainder of the oocytes at this time point were at GVBD (29.3% ± 4.7), MI (6.6% ± 2.9) or MII (1.2% ± 1.2). Only one oocyte was at the MII stage immediately after collection. The percentage of degenerated oocytes at this time point was significantly less than the percentage degenerating by 24 or 48 hours of culture ($P < 0.05$).

After 24 hours of culture, the majority of the oocytes had developed to the GVBD stage (41.3% ± 4.7) (Fig 2.6). Almost half of the remaining oocytes, however, were underdeveloped for this culture interval or had degenerated by

this time (30.4% ± 5.8 were at GV and 13.4% ± 3.5 had degenerated). The percent of oocytes maturing to MI after 24 hours of culture was 14.0% ± 3.1 and the percent maturing to MII was 0.9% ± 0.9.

After 48 hours of culture the percentage of oocytes maturing to the MI or MII stages had increased to 24.3% ± 3.5 and 17.0% ± 4.1 respectively (Fig 2.6). The percentage of oocytes maturing to the MI stage at this time point was significantly higher ($P < 0.05$) than the percentage reaching MI at the 0 hour stage. Additionally, the percentage reaching the MII stage was significantly higher ($P < 0.05$) than the percentage doing so at 0 or 24 hours of culture. The remaining oocytes at this time point were either immature after 48 hours of culture (25.3% ± 4.8 were GV and 18.5% ± 3.2 were GVBD) or had degenerated (15.4% ± 3.6).

Figure 2.6 Nuclear Status of dog oocytes cultured for 0, 24, or 48 hr. Different letters within the same meiotic stage indicate significant differences ($P < 0.05$). GV, germinal vesicle stage; GVBD germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

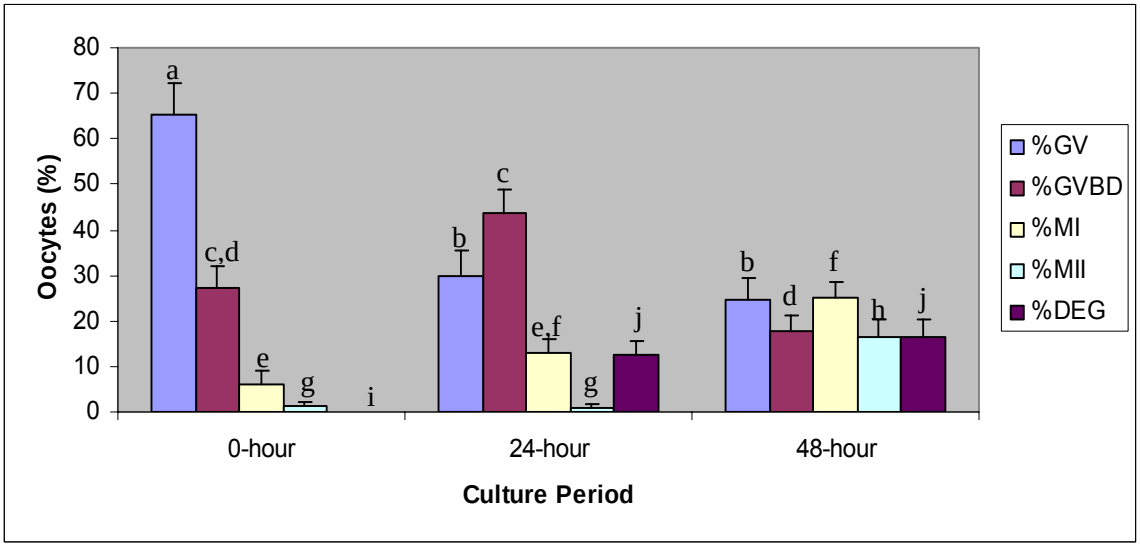


Table 1: Number of oocytes correlating to data represented in Figure 2.6

Culture Period	GV	GVBD	MI	MII	DEG
0	81	38	11	1	0
24	74	95	36	2	25
48	70	62	76	45	44

Looking individually at each culture interval, nuclear status of oocytes from different sized follicles was compared (Fig. 2.7-2.9). No statistically significant differences were found between follicle sizes in regard to the percentage of oocytes reaching MI/MII. The lack of statistical significance was likely due to the small sample size of oocytes collected from large follicles (n = 48) versus oocytes collected from small (n = 252) or medium (n = 231) follicles.

Separate analysis of the same data showed that significant differences within follicle sizes at various culture periods were present (Fig. 2.7-2.9). At the 0 hour culture interval small follicles had significantly more oocytes at the GV stage than at the MI or MII stage or that had degenerated ($P < 0.05$). Medium follicles at this time also had significantly more oocytes with a GV nuclear status than with MI, MII or degenerate status ($P < 0.05$). Oocytes from large follicles at 0 hours of culture showed no significant differences between meiotic stages.

At the 24 hour culture interval there were significantly more oocytes from small follicles at the GVBD stage than at MI or MII ($P < 0.05$). There were also significantly more oocytes at the GV stage than there were at MII ($P < 0.05$). This was also true for oocytes from medium follicles at this time, as there were significantly more oocytes with a GVBD nuclear status than with MII or degenerate status ($P < 0.05$) and there were significantly more oocytes at the GV stage than at the MII stage ($P < 0.05$). Large follicles at 24 hours of culture also had significantly more oocytes at the GVBD stage than at the MII stage ($P < 0.05$).

After 48 hours of culture, oocytes showed no statistically significant differences between meiotic stages within small, medium or large follicular sizes.

Figure 2.7 Nuclear status of dog oocytes from different sized follicles cultured for 0 hours. Different letters within the same follicle size indicate significant differences (P < 0.05). GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

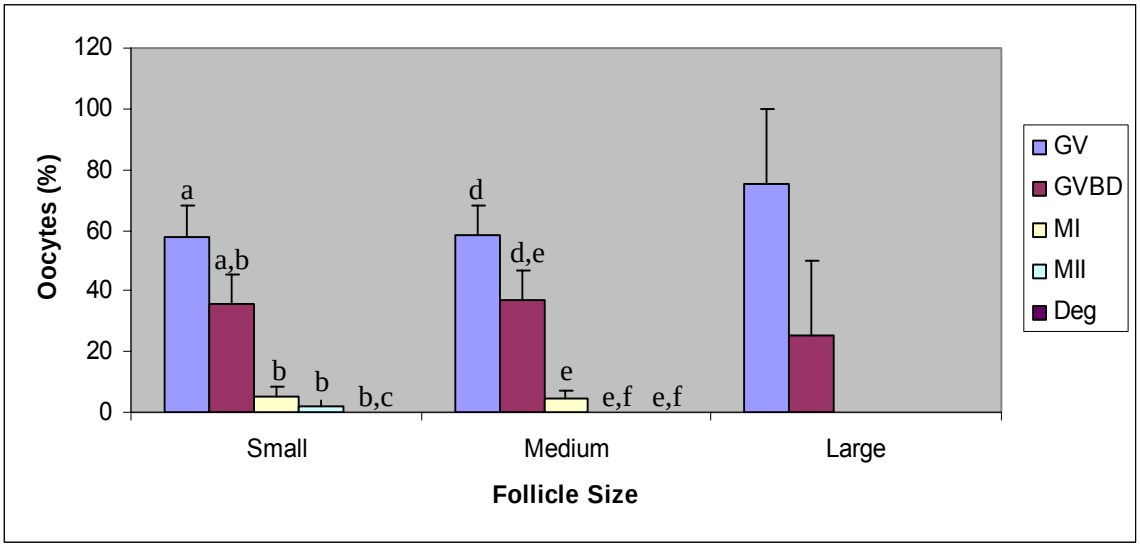


Figure 2.8 Nuclear status of dog oocytes from different sized follicles after 24 hours of culture. Different letters within the same follicular size indicate significant (P < 0.05) differences.

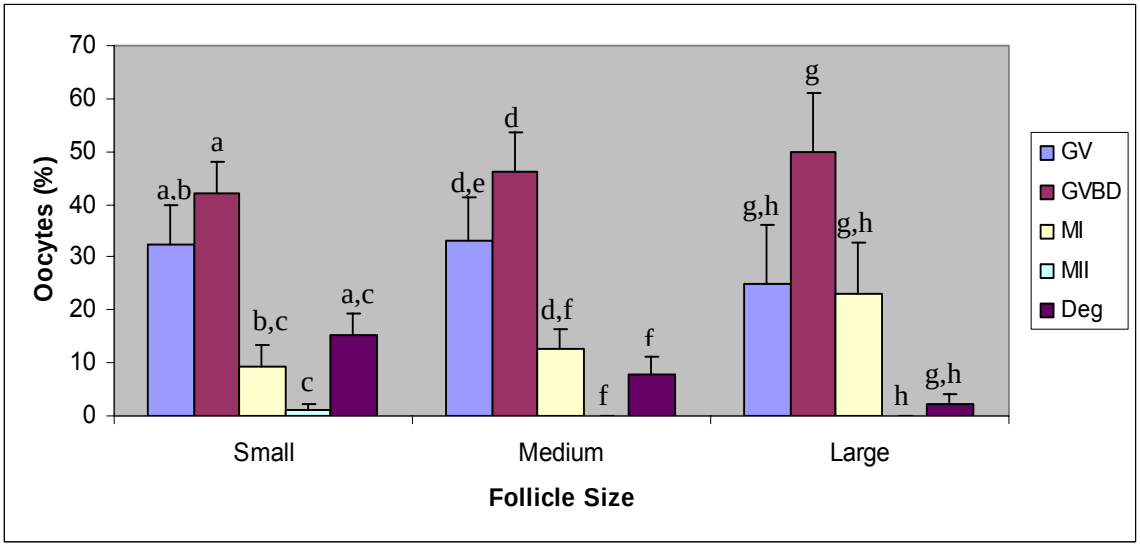


Figure 2.9 Nuclear status of dog oocytes from different sized follicles after 48 hours of culture. Different letters within the same follicular size indicate significant (P < 0.05) differences.

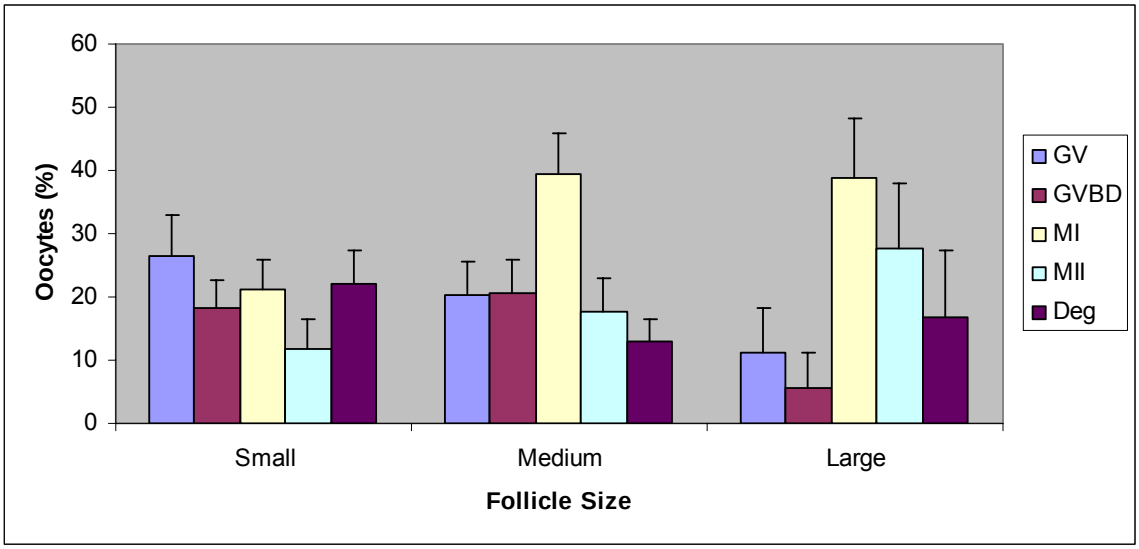


Table 2 Number of oocytes correlating to data represented in Figures 2.7-2.9

Culture Period	Follicle Size	GV	GVBD	MI	MII	DEG
0 hour	Small	36	21	4	1	0
	Medium	47	18	7	0	0
	Large	6	1	0	0	0
24 hour	Small	40	48	13	2	15
	Medium	30	39	16	0	9
	Large	4	8	7	0	1
48 hour	Small	41	31	35	15	25
	Medium	27	30	30	23	16
	Large	2	1	11	7	3

Influence of Follicle Size on Metabolic Rate

Metabolic rates of oocytes were significantly influenced by the size of follicle the oocyte was recovered from (Table 3). Across culture intervals and meiotic stages, oocytes recovered from large follicles metabolized significantly more pyruvate ($P < 0.05$) and glutamine ($P < 0.05$) than those recovered from small follicles. In addition, oocytes from large follicles had a higher glycolytic rate ($P < 0.05$) than oocytes recovered from small or medium follicles. Pyruvate uptake and glutamine metabolism of oocytes recovered from medium follicles was not significantly different from that of oocytes recovered from large follicles. The rate of glucose oxidation was not impacted by follicular size.

Table 3. Energy metabolism by dog oocytes of various follicular sizes (pmol/oocyte/hr; mean values ± SEM).

Follicle Size	[D-5^{3}H] Glucose (pmol/oocyte/hr)	[D-6^{14}C]Glucose (pmol/oocyte/hr)	[1-^{14}C] Pyruvate (pmol/oocyte/hr)	[^{3}H] Glutamine (pmol/oocyte/hr)
Small	2.11 ± 0.4 (82)[a]	0.31 ± 0.1 (82)	0.99 ± 0.1 (93)[a]	0.70 ± 0.2 (93)[a]
Medium	4.02 ± 0.7 (86)[b]	1.20 ± 0.5 (86)	1.12 ± 0.2 (79)[a,b]	1.58 ± 0.4 (79)[a,b]
Large	12.02 ± 3.4 (24)[c]	1.01 ± 0.7 (24)	1.23 ± 0.5 (15)[b]	2.52 ± 0.7 (15)[b]

[a,b] Different letters within the same column indicate significant differences ($P < 0.05$).

Influence of Maturation Time on Metabolic Rate

Across meiotic stages and follicle sizes, glycolytic rate was lowest in oocytes cultured for 24 hours ($P < 0.05$) compared to 0 or 48 hours (Table 4). There was not a significant difference between glycolytic rate of oocytes cultured for 0 hours or 48 hours ($P > 0.05$). Similar comparison across meiotic stages and follicular sizes revealed that the duration of culture did not significantly influence the rate of glucose oxidation, glutamine oxidation, or pyruvate uptake.

Table 4. Metabolism by dog oocytes at various culture intervals. (pmol/oocyte/hr; mean values ± SEM).

Maturation Time	[D-5^{3}H] Glucose (pmol/oocyte/hr)	[D-6^{14}C]Glucose (pmol/oocyte/hr)	[1-^{14}C] Pyruvate (pmol/oocyte/hr)	[^{3}H] Glutamine (pmol/oocyte/hr)
0	3.45 ± 1.1 (61)[a]	0.80 ± 0.3 (61)	1.18 ± 0.2 (68)	0.82 ± 0.2 (68)
24	2.89 ± 1.1 (68)[b]	0.98 ± 0.6 (68)	0.74 ± 0.2 (62)	1.44 ± 0.4 (62)
48	6.36 ± 0.8 (63)[a]	0.60 ± 0.3 (63)	1.29 ± 0.3 (57)	1.45 ± 0.4 (57)

[a,b] Different letters within the same column indicate significant differences (P < 0.05).

Influence of Meiotic Stage on Metabolic Rate

Nuclear status of oocytes had a significant impact on glycolytic rate. Oocytes at the MII stage had a significantly higher glycolytic rate than those at GV, GVBD or MI (P < 0.05) (Table 5). Nuclear status of oocytes did not significantly impact the rate of pyruvate uptake, glutamine oxidation or glucose oxidation.

Table 5. Metabolism by dog oocytes of various nuclear statuses. (pmol/oocyte/hr; mean values ± SEM).

Nuclear Status*	[D-5^{3}H] Glucose (pmol/oocyte/hr)	[D-6^{14}C]Glucose (pmol/oocyte/hr)	[1-^{14}C] Pyruvate (pmol/oocyte/hr)	[^{3}H]Glutamine (pmol/oocyte/hr)
GV	3.23 ± 1.5 (40)[a]	0.74 ± 0.4 (40)	1.47 ± 0.3 (39)	0.76 ± 0.2 (39)
GVBD	2.83 ± 1.0 (69)[a]	0.41 ± 0.1 (69)	0.86 ± 0.2 (61)	1.31 ± 0.5 (61)
MI	4.47 ± 1.0 (58)[a]	1.38 ± 0.8 (58)	1.09 ± 0.2 (64)	1.44 ± 0.3 (64)
MII	8.96 ± 0.8 (25)[b]	0.60 ± 0.2 (25)	0.86 ± 0.4 (23)	1.09 ± 0.5 (23)

[a,b] Different letters within the same column indicate significant differences (P < 0.05).

*GV, germinal vesicle stage; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

Substrate Utilization

Comparison of energy substrate utilization based on culture interval and follicle size was also done (Figs 2.10-2.12). Oocytes recovered from small follicles utilized pyruvate just as much as glycolysis or glutamine oxidation immediately after release from the follicle. The pyruvate uptake at this culture

interval was significantly higher than glucose oxidation ($P < 0.05$). Oocytes recovered from medium follicles, on the other hand, utilized glycolysis significantly more than pyruvate at the 0 hour culture interval ($P < 0.05$). The same trend seemed to apply to oocytes from large follicles at the 0 hour culture interval; however, the statistical differences were not significant.

Substrate utilization at the 24 hour culture period was not statistically different between oocytes recovered from small, medium or large follicles. However, several significant differences were found at the 48 hour culture period. Oocytes recovered from small follicles at this time point metabolized more glucose (via glycolysis) than glutamine and pyruvate ($P < 0.05$). Analysis of oocytes from medium follicles revealed that they also primarily utilized glycolysis. The glycolytic rate of these oocytes was significantly higher than the rate of glucose oxidation or glutamine oxidation ($P < 0.05$). Oocytes from large follicles followed the same trend, metabolizing significantly more glucose via glycolysis than they oxidized glucose or pyruvate ($P < 0.05$).

Figure 2.10 Energy substrates utilized by dog oocytes collected from small follicles at various culture periods. Different letters within the same culture period indicate significant differences ($P < 0.05$).

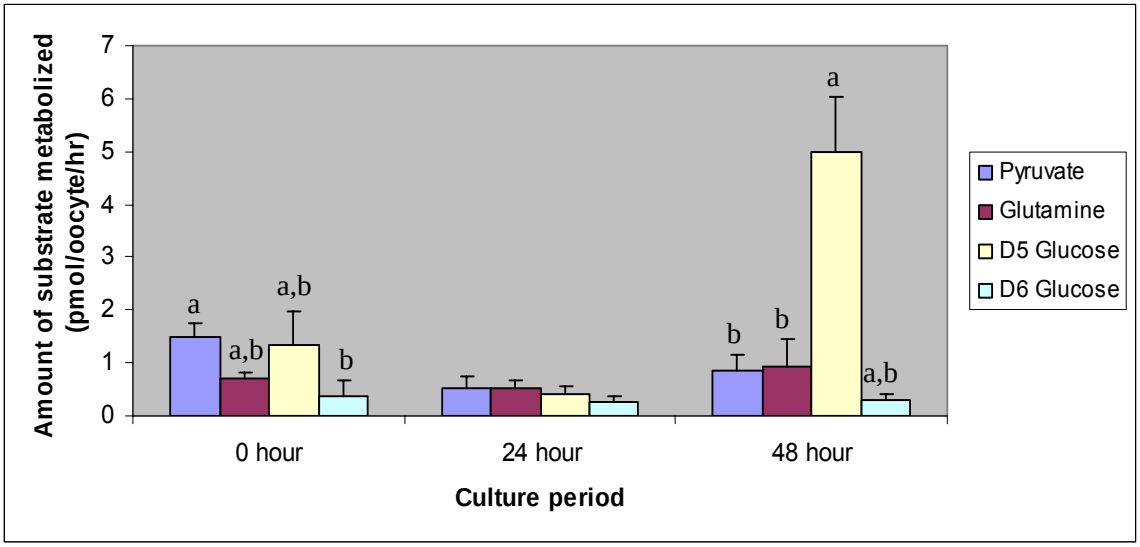


Figure 2.11 Energy substrates utilized by dog oocytes collected from medium follicles at various culture periods. Different letters within the same culture period indicate significant differences (P < 0.05).

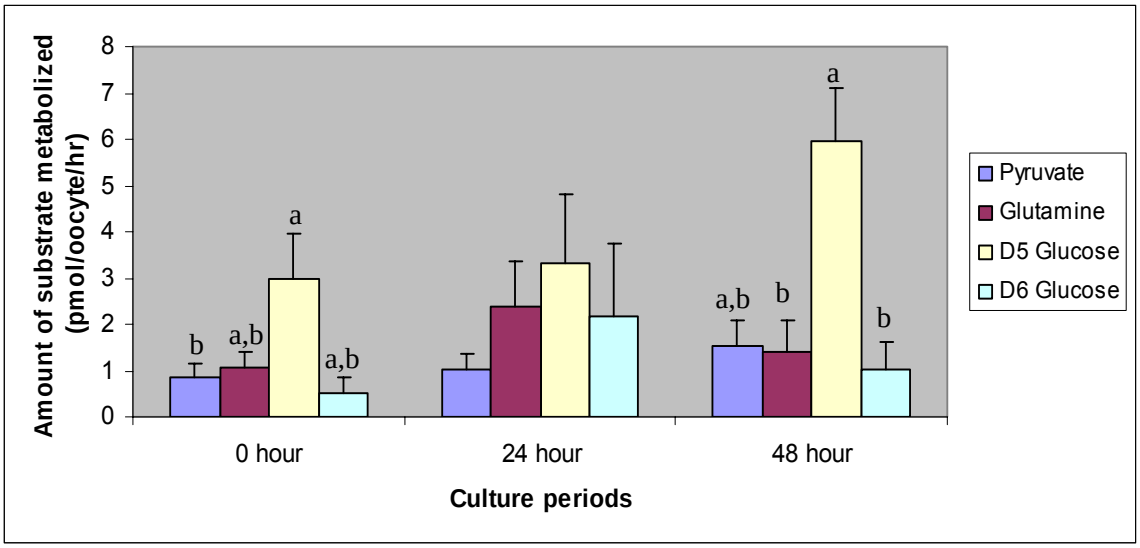


Figure 2.12 Energy substrates utilized by dog oocytes collected from large follicles at various culture periods. Different letters within the same culture period indicate significant differences (P < 0.05).

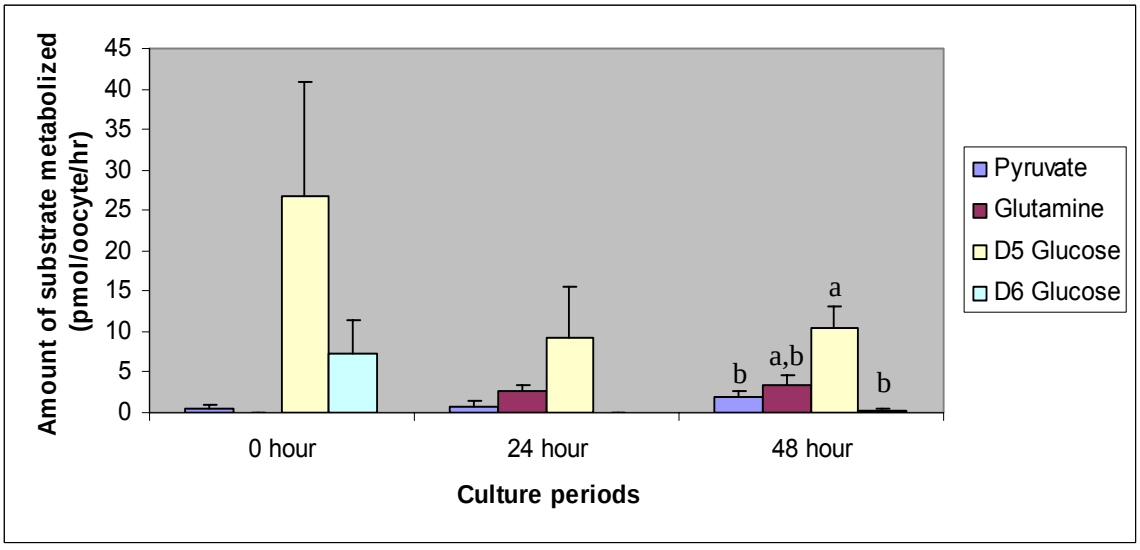


Table 6 Number of oocytes correlating to data represented in Figures 2.10-2.12

Follicle Size	Substrates Analyzed	0 hours culture	24 hours culture	48 hours culture
Small	Pyruvate/Glutamine	36	32	25
	D5/D6 Glucose	26	31	25
Medium	Pyruvate/Glutamine	29	25	25
	D5/D6 Glucose	32	27	27
Large	Pyruvate/Glutamine	3	5	7
	D5/D6 Glucose	3	10	11

Discussion

Maturation Kinetics

The overall maturation rate of dog oocytes in vitro to the MI or MII stage achieved in this study was 25.8%. This is similar to results achieved in previous IVM studies with dog oocytes (see Chapter 1). As expected, most oocytes were at the GV stage immediately after release from the follicle. There were however, 11 oocytes at the MI stage and one oocyte that had already reached MII at the 0 hour culture interval. Seeing oocytes at these advanced stages at 0 hours of culture is supportive of the capability of canine oocytes to undergo parthenogenetic activation within the follicle. The presence of activated canine oocytes up to the 4-cell embryo stage immediately after oocyte collection via slicing has been observed (N.Songsasen, personal communication, 2007). Many mammalian oocytes have been shown to undergo parthenogenetic activation in vitro after exposure to a variety of stimuli such as electrical impulses, temperature shock, and hyaluronidase treatment.[78] In rats, it appears that oxygen deprivation may be a sufficient stimulus to induce parthenogenetic activation.[79] In vivo parthenogenetic activation has also been documented in a particular strain of inbred mice.[80] Approximately 10% of the oocytes of this inbred mouse line activated parthenogenetically in vivo after ovulation, with most of the oocytes developing normally for 5 or 6 days before becoming disorganized

and degenerating.[80] This type of spontaneous activation in vivo is considered a rare occurrence in mammalian species and is thought to occur at the end of the fertilizable lifespan of the oocyte.[81] It may reflect an inability to maintain the meiotic block and be an indication of the first stages of oocyte degeneration.[81] The significance of spontaneous oocyte activation in the domestic dog has yet to be determined; however, it is interesting to note that this phenomenon can and does occur in the canine.

By 24 hours of culture most oocytes had resumed meiosis and progressed to the GVBD stage, with several other oocytes already reaching MI or MII. It was at the same time point that the inefficiency of IVM protocols in the canine became evident. The percentage of oocytes that had degenerated or failed to mature beyond the GV stage was at 43.8%. After 48 hours in culture, the percentage of oocytes that had matured to MI or MII had increased; yet, the percentage of degenerate or immature oocytes had increased as well, now nearly 60%. These discouraging maturation rates provide additional proof that current IVM protocols in the dog are inefficient to say the least.

One promising advance in canine IVM may relate to selection of oocytes from follicles greater than 2 mm in diameter, as previous research has demonstrated that dog oocytes collected from such follicles reach meiotic competency at substantially greater rates than oocytes collected from follicles that are smaller in diameter (80% compared to 38.4% in follicles from 1 to 2 mm, 26.1% in follicles from 0.5 to 1 mm, and 16.9% in follicles less than 0.5 mm).[52] In the present study, oocytes collected from large follicles did not have statistically greater rates of maturation to the MI/MII stage compared to oocytes collected from small follicles. This lack of statistical difference may be a result of the relatively small sample size of oocytes recovered from large follicles (n = 48) versus oocytes collected from small (n = 252) or medium (n = 231) follicles.

Metabolic Activity of the Dog Oocyte

The goal of this study was to contribute to the overall knowledge regarding metabolism of domestic dog oocytes in vitro by elucidating species-specific metabolic activities. The ability to supplement the proper energy substrates at the ideal times and concentrations has the potential to vastly improve current IVM protocols in the canine. Analysis of the metabolic activity of oocytes during development is important because changes in metabolic rate have been correlated to changes in oocyte maturation.[67,82-84] It has also been shown that the type of energy substrates present during oocyte culture in vitro alters both the metabolic activity and the subsequent developmental competence of those oocytes.[66,82,85] Thus, elucidation of metabolic activity specific to domestic dog oocytes will play a large role in further IVM success.

As mentioned above, research has shown that oocytes collected from larger follicles have an increased ability to complete maturation.[52] The present study has demonstrated that in addition to increased rates of maturation, oocytes recovered from large follicles (> 2 mm in diameter) also have increased metabolic rates compared to oocytes collected from smaller follicles (< 1 mm in diameter). Glycolysis, pyruvate oxidation and glutamine oxidation were all utilized significantly more by oocytes from large follicles than by oocytes from small follicles. The fact that both metabolic rate and developmental competence have been linked to the size of donor follicles could have substantial implications for oocyte selection in future IVM protocols. It may be beneficial to begin excluding oocytes from in vitro culture that are found in follicles less than 1 mm in size in vivo.

Of the four substrates analyzed in this study, denuded dog oocytes seemed to preferentially utilize glucose (via glycolysis) as an energy source during the 3 hour IVM period that was assessed. Compared to other species, this appears to be a very unique finding. In the mouse, denuded oocytes provided only glucose during 16-19 hours of IVM are incapable of reaching maturation, requiring supplementation with either pyruvate or oxaloacetate instead.[71] The same is true of denuded oocytes in the cow, which also require

pyruvate as a primary energy source during 24 hours of IVM.[86] Even the denuded oocytes of another carnivore, the cat, preferentially utilize pyruvate over glucose throughout IVM.[66] It has been suggested that mammalian oocytes utilize energy substrates via the surrounding cumulus cells. Specifically, the cumulus cells metabolize glucose into pyruvate or other intermediates of the Krebs cycle that can be passed via gap junctions to the oocyte to serve as a source of energy.[72] This is also the method by which adenosine triphosphate (ATP), another source of energy, is hypothesized to gain access to the oocyte, as it is not taken up efficiently by the oocyte plasma membrane itself.[72] It seems that the dog oocyte, on the other hand, is able to efficiently provide energy for itself by metabolizing glucose without the aid of any surrounding cumulus cells.

In addition to preferentially utilizing glycolysis over other energy substrates, dog oocytes increasingly relied on glycolysis as they matured from the GV stage up to the MII stage. In fact, oocytes at the MII stage had a significantly higher glycolytic rate than oocytes at the GV, GVBD or MI stage. This suggests that increasing rates of glycolysis correlate well to increasing stages of maturation in the dog oocyte. In the cat oocyte, although glycolysis is not the predominant metabolic pathway, increased glycolytic rate did predict which oocytes would have the highest rate of blastocyst development after fertilization.[66] Oocytes of the cow showed similar tendencies, as increased glycolytic rate has been associated with increased developmental competency in this species as well.[82] With the apparent importance of glycolysis in the maturation of dog oocytes, perhaps increased glycolytic rate implies increased capacity for embryonic development after fertilization as it does in the cat and cow. Further study would be necessary to confirm whether such a correlation exists.

In contrast to the striking role of glucose utilization via glycolysis, glucose utilization via oxidation does not appear to play a large role in metabolism of the dog oocyte. Oxidation of [6-^{14}C] glucose remained at fairly low levels throughout all culture periods and its use did not significantly vary between oocytes from any follicular size or meiotic stage. The reason why dog oocytes appear to utilize

glucose in a predominantly anaerobic fashion, bypassing the potential for increased energy yield that oxidative metabolism can provide, may be linked to the in vivo oxygen tension of the reproductive tract. Research has indicated that the oxygen tension in the mammalian reproductive tract is much lower than atmospheric oxygen tension (approximately 5% O_2 versus 20% O_2).[87] It is possible that after glucose is converted to pyruvate via glycolysis, pyruvate is being converted to lactate and subsequently exported from the cell instead of entering the Kreb's cycle and undergoing oxidative metabolism. This phenomenon has been documented in mice[88], sheep[89] and cattle[90] embryos, but has never been documented in oocytes. Measurement of lactate production in future dog oocyte studies might shed light on this possibility.

As was the case with glucose metabolism in the dog oocyte, the role of pyruvate metabolism in this species also seems to be unique. Previous research has indicated that pyruvate supplementation failed to enhance nuclear maturation of the denuded dog oocyte.[77] The fact that pyruvate does not seem to play a leading role in resumption or completion of nuclear maturation in the dog oocyte sets it apart from metabolic patterns established for other species. Supplementation of pyruvate has been shown to play a crucial role in the resumption of meiosis and subsequent nuclear maturation in the mouse oocyte.[74,91] In the cat oocyte, pyruvate was found to be the preferred substrate during oocyte development at all stages.[66] This was also found to be the case in cattle[67], rabbit[92], and rhesus monkey (*Macaca mulatta*)[93] oocytes.

In the present study, comparison of pyruvate metabolism between oocytes of various meiotic stages showed no significant difference in uptake of this substrate. Furthermore, no significant differences between pyruvate uptake by oocytes at 0, 24 or 48 hours of culture were noted. This appears to contrast previous research that indicated pyruvate seemed to play a significant role in resumption of meiosis from the GV to the GVBD stage, after which utilization of this substrate dropped off.[77] Perhaps pyruvate does not play as large of a role in resumption of meiosis as previously thought. Interestingly, analysis of pyruvate metabolism across all culture intervals showed that pyruvate uptake was

significantly higher in oocytes recovered from large follicles than it was in oocytes recovered from small follicles. This finding may point to an increased metabolic role for pyruvate uptake in oocytes with greater developmental capacity. It was also noted that oocytes recovered from small follicles utilized pyruvate at a rate equal to the rate that glycolysis was used at 0 hours of culture. Comparatively, oocytes from medium ($P < 0.05$) and large ($P > 0.05$) follicles at 0 hours of culture predominantly utilized glycolysis over pyruvate. This may suggest that oocytes recovered from small follicles have such low metabolic activity, especially with regard to glucose, that no appreciable differences in substrate utilization are recognizable.

Analysis of glutamine metabolism in the dog oocyte has also revealed patterns that vary considerably from other species. Previous research indicated that the most significant amount of glutamine oxidation occurred at 12 hours of culture, correlating to a time when most oocytes were at the GVBD stage.[77] By 36 hours of culture glutamine oxidation had dropped significantly, suggesting that the role of this substrate was only associated with the resumption of meiosis in the dog oocyte. This pattern was different from oocytes of the cow which showed increases in glutamine oxidation around the time that oocytes completed nuclear maturation.[67,94] Oocytes of the cat, in contrast, appeared to utilize increasing amounts of glutamine until completion of GVBD, after which metabolism of this substrate remained at constant levels instead of dropping off (as seen in the dog).[66]

In the current study, glutamine metabolism did not vary significantly between oocytes at 0, 24, or 48 hours of culture, nor did it vary significantly between oocytes of different meiotic stages. Perhaps the fact that metabolic activity was not assessed at the 12 hour time point in this study caused a peak in glutamine metabolism to go unnoticed. The importance of such a peak, if it was present, may not be of overall significance, however, as previous research has indicated that supplementation of glutamine to the culture medium does not have a significant impact on nuclear maturation in dog oocytes.[77] This is in contrast to oocytes of the rabbit,[95] cow,[94,96] and hamster (*Mesocricetus auratus*)[97] in which

glutamine supplementation to culture medium enhances nuclear maturation. Conversely, when mouse oocytes are added to culture medium supplemented with glutamine the resumption of meiosis appears to be suppressed.[98]

Despite the fact that glutamine may not significantly impact nuclear maturation in dog oocytes, this amino acid was metabolized at all culture intervals, and by oocytes of each meiotic stage in the study. It was also metabolized at a significantly higher rate by oocytes recovered from large follicles which have been proven to complete maturation at increased rates. This may suggest that while the presence of glutamine in culture media is not required for nuclear maturation, it may play a role (as pyruvate seems to) in increasing the metabolic rate and subsequent nuclear maturation in oocytes of superior developmental competence.

In addition to analysis of carbohydrate and amino acid supplementation to culture medium, analysis of lipid metabolism may also be required to create optimum conditions for canine IVM. Just like the cat and the cow, the dog oocyte contains a significant amount of intracellular lipid.[99-101] The presence of large amounts of intra-oocyte lipid may provide a significant energy reserve to the dog oocyte, like it does in the pig oocyte.[102] Perhaps a unique ability to rely on this energy reserve is responsible for some of the interesting differences seen in carbohydrate metabolism in dog oocytes versus oocytes in other species.

While evaluation of individual energy substrates can shed light on the metabolic activity of oocytes, it is generally accepted that supplementation with a combination of energy substrates is required for mammalian oocytes to undergo nuclear maturation in vitro.[85,98] It is therefore necessary to establish a culture system with not only the right substrates present, but also the right combination of substrates present. For instance, although glucose has been implicated in this study as the preferred energy substrate for the dog oocyte, previous research showed that dog oocytes cultured in media with a high glucose concentration and an absence of pyruvate resulted in decreased development to the MII stage.[41] Thus, even though pyruvate has since been deemed inessential for nuclear maturation of the dog oocyte, it appears that supplementation of some sort in

addition to glucose is necessary to achieve maximal rates of maturation. Further research comparing the effects of varying levels of energy substrates in addition to baseline levels of glucose are warranted to determine the most effective combination of energy substrates for canine IVM. In addition, the results of this study suggest that the size of follicle an oocyte is collected from may be of utmost importance in predicting the capacity for overall metabolic rate and subsequent developmental competence. Research focusing on oocytes selected solely from medium or large follicles could help separate out extraneous data collected from oocytes of small follicles that cannot metabolize or mature as efficiently as their larger counterparts. This could allow culture protocols based only on requirements for the most metabolically capable oocytes to be elucidated.

Conclusion

In conclusion, oocytes of the domestic dog appear to display species specific patterns of metabolic activity. The ability of dog oocytes denuded of cumulus cells to preferentially utilize glucose as an energy substrate is unique among domestic species studied to date. The present data results suggest that increasing glycolytic rate correlates well to increasing stages of maturation. In addition, this study has demonstrated that oocytes collected from follicles greater than 2 mm in diameter have increased metabolic capabilities. This realization lends itself to the establishment of more exclusive selection criteria for future canine IVM studies. Overall, oocyte metabolism appears to be yet another example where the domestic dog stands apart from its fellow domestic species.

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Appendix A - Source of Supplies

Source of supplies used:

Company	Item	Catalog Number
Fisher Scientific (81 Wyman Street, Waltham, MA 02454)	50 ml Centrifuge tube	430290
	15 ml Disposable sterile centrifuge tube	05-539-5
	Falcon 35 x 10 mm petri dishes	1008
	Flex-tube 1.5 ml microcentrifuge tubes	22-36-411-1
	2.5 L Acetic acid, glacial	A38-212
	8 mL Scintillation vials	5004A.M00.002
	Multidish 4 wells	176740
	Rainin 250 µL pipette tips	RT-250
	Rainin 1000 µL pipette tips	RT-200
	5 3/4” Disposable Pasteur pipettes	13-678-20B
	Ethyl alcohol 200 proof, 1 pint	64-17-5
Sigma-Aldrich (3050 Spruce St., St. Louis, MO 63103)	Mineral oil	M-8410
	Medium 199- HEPES Modification (500ml)	M-7528

Sigma, cont.	Medium 199 (500ml)	M-2154
	2-Mercapto-ethanol	M-7522
	Penicillin G potassium salt	P4687-10MU
	L-Glutamine	G-8540
	Polyvinyl alcohol	P-8136
	Sodium bicarbonate	S-5761
	Albumin, bovine, 96-99%	A3311-50G
	Pyruvic acid	P-4562
	Streptomycin sulfate	S-9137
	Equine chorionic gonadotropin	G-4527
	Epidermal growth factor	E-4127
	Orcein (Natural red 28), 5g	0-7505
	Glycerol, 500 mL	G-2025
GE Health (2655 Dividend Dr 117, Memphis, TN 38132)	[1- 14C] Pyruvic acid, sodium salt	CFA 85
	D-[6-14C] Glucose	CFA 351
	D-[5-3H] Glucose	TRK 290
	L-[G-3H] Glutamine	TRK 459
Perkin Elmer Life Sciences (2200 Warrenville Rd., Downers Grove, IL 60515)	Ultima Gold (4 x 2.5 L)	6013327
Rainin (7500 Edgewater Dr., Oakland, CA 94621)	200 µL pipette	B0615424A
	20 µL pipette	B0614169A
	1000 µL pipette	B0614169A

Appendix B - Media Recipes

Canine IVM Media

TCM 199 (Earle's salt)	100 mL
Pyruvic acid	0.0028 g
L-glutamine	0.0292 g
Penicillin G potassium salt	0.003 g
Streptomycin sulfate	0.0003 g
Polyvinyl alcohol	0.1 g

After preparation, osmolality was adjusted to 285-295 with Milli-Q water. Media was prepared weekly.

Canine Collection Media

TCM + Hepes	200 mL
Pyruvic acid	0.005 g
L-glutamine	0.058 g
Penicillin G potassium salt	0.006 g
Streptomycin sulfate	0.006 g
Bovine serum albumin	0.6 g

Media was prepared on a weekly basis.

Appendix C – Expanded Data Tables

Energy metabolism by dog oocytes of various follicular sizes, maturation times, and nuclear statuses (pmol/oocyte/hr, mean values ± SEM).

Follicle size	**Maturation time (hr)**	**Number of oocytes**	**Nuclear status***	**[D-5^{3}H] Glucose (pmol/oocyte/hr)**	**[D-6^{14}C]Glucose (pmol/oocyte/hr)**
Small	0	15	GV	0.4 ± 0.3	0.1 ± 0
		10	GVBD	2.4 ± 1.2	0.8 ± 0.6
		1	MI	0	0
	24	28	GVBD	0.3 ± 0.1	0.2 ± 0.1
		3	MI	1.0 ± 0.6	0 ± 0
	48	15	MI	2.1 ± 0.9	0.1 ± 0
		10	MII	7.4 ± 1.1	0.5 ± 0.2
Medium	0	22	GV	1.6 ± 0.7	0.3 ± 0.2
		8	GVBD	2.9 ± 1.8	0.1 ± 0.1
		2	MI	10.6 ± 7.1	3.8 ± 3.8
	24	18	GVBD	2.0 ± 0.5	0.5 ± 0.3
		9	MI	4.5 ± 3.7	4.5 ± 4.0
	48	15	MI	3.5 ± 1.3	1.1 ± 0.9
		12	MII	6.9 ± 1.3	0.5 ± 0.3
Large	0	2	GV	13.3 ± 13.3	6.2 ± 6.2
	24	2	GVBD	2.4 ± 0.6	0
		5	MI	1.1 ± 0.6	0 ± 0
	48	6	MI	3.5 ± 0.8	0 ± 0
		3	MII	11.0 ± 1.5	0.5 ± 0.5

*GV, germinal vesicle stage; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

Energy metabolism by dog oocytes of various follicular sizes, maturation times, and nuclear statuses (pmol/oocyte/hr, mean values ± SEM).

Follicle size	**Maturation time (hr)**	**Number of oocytes**	**Nuclear status***	**[1-^{14}C] Pyruvate (pmol/oocyte/hr)**	**[^{3}H]Glutamine (pmol/oocyte/hr)**
Small	0	21	GV	1.8 ± 0.3	0.7 ± 0.2
		11	GVBD	0.8 ± 0.3	0.5 ± 0.2
		3	MI	1.9 ± 0.7	1.2 ± 0.5
		1	MII	0.8	0.3
	24	20	GVBD	0.5 ± 0.3	0.4 ± 0.2
		12	MI	0.5 ± 0.3	0.8 ± 0.3
	48	18	MI	1.2 ± 0.4	0.9 ± 0.6
		7	MII	0	1.1 ± 1.1
Medium	0	16	GV	1.1 ± 0.4	0.9 ± 0.3
		8	GVBD	0.9 ± 0.8	1.7 ± 1.2
		5	MI	0	0.5 ± 0.4
	24	18	GVBD	1.2 ± 0.5	2.5 ± 1.4
		7	MI	0.5 ± 0.5	1.9 ± 0.8
	48	14	MI	1.7 ± 0.8	2.1 ± 1.1
		11	MII	1.4 ± 0.7	0.4 ± 0.3
Large	0	2	GV	0.5 ± 0.5	0
		1	GVBD	0.6	0.1
	24	3	GVBD	1.1 ±1.1	2.7 ± 1.0
		2	MI	0 ± 0	2.8 ± 1.4
	48	3	MI	3.1 ± 0.8	3.8 ± 1.8
		4	MII	1.0 ± 0.8	3.2 ± 2.1

*GV, germinal vesicle stage; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.