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In vitro development of bovine embryos cultured in a frozen-thawed commercial culture medium

Jeanne Lee Glaser
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IN VITRO DEVELOPMENT OF BOVINE EMBRYOS CULTURED IN A FROZEN-THAWED COMMERCIAL CULTURE MEDIUM

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in

The Interdepartmental Program of Animal and Dairy Sciences

by

Jeanne Lee Glaser
B.S., Louisiana State University, 2003
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ART – assisted reproductive technologies
BME – basal medium Eagle
BSA – bovine serum albumin
CO2 – carbon dioxide
EAA – essential amino acids
EB – early blastocyst
EBL – Embryo Biotechnology Laboratory
ET – embryo transfer
FBS – fetal bovine serum
FSH – Follicle Stimulating Hormone
Hatched – hatched blastocyst
Hatching – hatching blastocyst
HECM – hamster embryo culture medium
IGF2 – insulin-like growth factor 2
IVC – in vitro culture
IVF – in vitro fertilization
K – Potassium
LDH – lactate dehydrogenase
LH – Luteinizing Hormone
MEM – minimum essential medium
mSOF – modified synthetic oviduct fluid medium
N2 – Nitrogen
Na – Sodium
NEA – nonessential amino acids
O2 – oxygen
PVA – polyvinylalcohol
TALP – Tyrode’s-albumin-lactate-pyruvate
TL HEPES – Tyrodes lactate HEPES
TCM-199 – tissue culture medium
XB – expanded blastocyst
ZIFT – zygote intrafallopian transfer
ABSTRACT

As in vitro fertilization (IVF) becomes more acceptable for the treatment of human infertility, it is essential for clinics to have in place an optimal embryo culture system. The objective of this study was to evaluate whether freezing and storing human embryo culture medium will alter embryo post-thaw viability. In consecutive experiments, commercial culture medium was frozen at -20°C and at -80°C and stored at 4°C until its use in IVF. Each IVF-derived replicate of bovine embryos contained a laboratory control group, a fresh commercial culture medium group(s) and a frozen-thawed commercial culture medium group(s). The commercial culture medium was supplemented with 15% of BSA for days 1 through 4 of in vitro culture and on day 4 was supplemented with 9% of glucose. Embryos were cultured to day 8 of the experiment and then embryo development was evaluated and morphology was evaluated using the RED Score system. In Experiment 1, zygotes (n=2,094) were in vitro cultured in medium that had been frozen (-20°C) with storage times ranging from 3 days to 10 weeks. It was found that embryo culture medium frozen at -20°C and stored for up to 10 weeks did not cause a decrease in 8- to 16-cell embryo or blastocyst development for those embryos cultured in medium frozen for 3 days to 6 weeks over the fresh medium (0 weeks). In Experiment 2, zygotes (n=3,727) were in vitro cultured in both fresh and frozen commercial culture medium (-80°C) that was stored for 0, 1 or 2 weeks before use. This study indicated embryo culture medium frozen at -80°C stored for up to 2 weeks did not cause a decrease in 8- to 16-cell embryo or blastocyst development over that of the fresh medium (0 week). In summary, it was concluded that there was no difference in the blastocyst development rate or morphology at the end of the culture period in medium frozen at -20°C for up to 10 weeks or in medium frozen at -80°C for up to 2 weeks.
CHAPTER I
INTRODUCTION

In the second half of the 20th century, there was development and advancement in controlled culture techniques for early stage mammalian embryos leading to the first in vitro fertilization (IVF) offspring of several species, including humans. Due to the development of improved in vitro culture conditions, murine offspring were reported from the transfer of in vitro cultured embryos (McLaren and Biggers, 1958). Whitten (1957) previously reported success in developing 8-cell mouse embryos to the blastocyst stage in vitro using a defined culture medium. In 1959, first time mammalian offspring (a rabbit) was reported from the transfer of in vitro fertilized embryos (Chang, 1959).

Decades prior to Whitten’s accomplishments, Albert Brachet was first to report keeping a rabbit blastocyst alive and developing for 48 hours outside the mother’s body in blood plasma (Brachet, 1912, 1913). Lewis and Gregory (1929) were able to film the development of rabbit eggs under the microscope on glass slides from the initial cleavage stages to the blastocyst stage (see Alexandre, 2001). Prior to this time, the basic culture medium used to incubate embryos was either blood plasma or serum.

In an effort to address the metabolic requirements of mouse embryos, Whitten (1957) and Brinster (1963) improved culture conditions to the extent that culturing from the 2-cell stage to the morula stage became relatively easy, while also being efficient and reproducible. Even with the early success of mouse embryo culture, Alexandre (2001) reports that scientists thought it was unimaginable to develop valid experimental strategies for culturing mammalian embryos without a serum- or a plasma-free medium, since the composition of these body fluids was complex and highly variable.

In 1978, the first human baby was produced by in vitro fertilization by biologist Robert G. Edwards and gynecologist Patrick Steptoe (Steptoe and Edwards, 1978). It was not until four years later that Bracket et al. (1982) reported the first ruminant offspring (a bull calf) from in vitro fertilization. Subsequently, the first IVF-derived offspring were reported in the two other domestic ruminants, goats (Hanada, 1985) and sheep (Cheng et al., 1986).
Today's quest has led to finding a more defined culture medium, without the variability of the components used by the early researchers such as Brachet and Lewis. Once a desired medium is decided upon there are quality control tests that must be performed. Most fertility clinics set up an animal model system to evaluate culture media, as well as perform a number of assays to test for various factors. A number of authors (Purdy, 1982; Trounson, 1982; Vijayakumar et al., 1987; Naaktgeboren, 1987; Davidson et al., 1988a, 1988b; McDowell et al., 1988) have suggested validation from the performance of an assay for mammalian embryo culture to control the suitability of a preparation of culture medium before using it for human IVF. For stronger results and less variation a number of in vitro replicates must be provided within the same culture medium.

Recommendations and regulations have been set regarding the storage time period for embryo culture media. The current procedures are to manufacture a new batch of media every 2 weeks (Purdy, 1982; Quinn et al., 1984; Gerrity et al., 1992; McLendon, 1992). Adhering to these guidelines creates a limited number of IVF replicates performed using a single batch of culture medium, reducing the data available for comparison. Replication among one single batch is important because gametes and embryos are being exposed to the same treatments. Due to variation between batches of culture media or variation between commercial culture media production laboratories, the use of one single batch of medium can assess embryonic development in vitro more clearly.

Extended refrigerator storage at 4°C or frozen storage of embryo culture media has been suggested to overcome this limited storage time barrier. Unfortunately, only a few investigators (Naz et al., 1986; Bernart et al., 1990; De Silva, 1993; Bell et al., 1995) have evaluated the effect of refrigerated or frozen stored culture media on embryo development and the frozen-thawed viability of components in embryo culture media.

The quality of the culture medium has been reported to be a crucial factor influencing the success of IVF in human infertility programs (Jones et al., 1982; Purdy, 1982; Trounson, 1982; Dandekar and Quigley, 1984; Quinn et al., 1984; McDowell et al., 1988). Therefore, the advantages of using the extended storage time created by refrigerator-stored or frozen-stored embryo culture media can be very beneficial. The number of assays, as well as IVF cycles performed, within one batch of medium can be
greatly increased. This would create a better understanding of the potential in vitro culture (IVC) ability of a particular medium.

With an increase in the number of human assisted reproductive treatment (ART) cycles performed in the United States almost doubling from 64,681 cycles in 1996 to 122,872 in 2003 (Centers for Disease Control and Prevention 2003 Assisted Reproductive Technology Success Rate Report), it is also important for commercial culture media companies to be able to efficiently produce a mass amount of media to keep up with the demand. This review will evaluate IVF-derived blastocyst production using fresh and frozen-thawed human commercial culture medium to culture bovine embryos.
CHAPTER II
LITERATURE REVIEW

In Vitro Fertilization of Mammalian Embryos

The process of mammalian in vitro fertilization (IVF) is the joining together of a male and female gamete outside of the body. For fertilization to occur the medium conditions must be optimal. There are a number of events that must take place before an embryo develops, resulting in implantation.

Oocyte Maturation

For fertilization to occur an acrosome reacted spermatozoan (sperm) enters a matured oocyte. During oocyte maturation there is progression of nuclear maturation from the germinal vesicle of the arrested dictyate stage, through meiosis I to metaphase II and extrusion of the first polar body (First and Parish, 1987). There are a few key factors that are important for complete oocyte maturation to occur. Moor and Gandolfi (1987) indicated that especially important are the development of proteins in the cytoplasm which regulate: meiotic events, sperm decondensation, formation of the male pronuclear envelope, initiation of cleavage, completion of cleavage-stage embryo development and the ability to produce a viable offspring.

All of these regulatory events can be affected by the duration of the in vivo or in vitro maturation time of the oocyte and the culture conditions during in vitro maturation. Common defects resulting from fertilization of immature oocytes include failure of male pronuclear development (Thibault et al., 1975; Thibault, 1977) and failure to progress to organized blastocyst stage embryos (Staigmiller and Moor, 1984; Leibfried-Rutledge et al., 1987).

Sperm Capacitation

Sperm must also go through a maturation process known as capacitation before the male gamete is able to fertilize an oocyte (Austin, 1951; Chang, 1951). This process normally takes place in the female reproductive tract. However, in the in vitro environment a culture condition must be recreated similar to that of the environment in vivo. Capacitation consists of two main functions, an initial sperm membrane alteration (Yanagimachi, 1981; Wolf et al., 1981; O'Rand, 1982; Ahuja, 1984; Langlais and Roberts, 1985), which allows the sperm to undergo the second phase, the acrosome
reaction (Yanagimachi and Usui, 1974). In mammalian species, sperm must begin to penetrate the cumulus and corona radiata with an intact acrosome. There is a release of enzymes from the acrosome of the sperm, such as hyaluronidase, starting the digestion of proteins in the zona pellucida enabling fertilization. The acrosome reaction of the sperm is then stimulated by the interaction of the contact between the sperm and the zona pellucida. Fusion of the plasma membrane occurs with the outer acrosomal membrane vesiculation thus, exposing the acrosome contents.

**Oocyte Activation and Fertilization**

In mammalian species, once the first sperm binds to the zona pellucida activation of that oocyte occurs. To prohibit polyspermy, penetration of the zona by more than one sperm, the oocyte responds with a cortical reaction. This cortical reaction produces a block to polyspermy fertilization. There is a massive exocytosis of cortical granules containing a mixture of enzymes released into the perivitelline space, which causes the zona pellucida to harden. Also the sperm receptors are lost preventing additional sperm from binding to the zona pellucida.

Once the individual sperm penetrates the zona pellucida, the sperm nucleus decondenses in the cytoplasm of the oocyte, resulting in the swelling of the sperm head forming the male pronucleus. Completion of meiosis II allows the formation of the female pronucleus to become complete and pronuclear fusion or syngamy occurs. Fertilization has occurred forming a zygote and then with mitosis the first cleavage of the new embryo (2-cell).

Completion of all of these steps is fundamental to successful in vitro fertilization. In addition, it should also be pointed out that sperm concentration, time of sperm-oocyte interaction, medium quality and temperature are all important in successful fertilization and for developmentally competent zygotes (First and Parrish, 1987).

The production of viable embryos from in vitro fertilization (IVF) is dependent on the availability of a suitable in vitro culture system capable of supporting developing embryos to the morula or blastocyst stage for transplantation. With optimal culture conditions the embryo undergoes compaction of the blastomeres followed by differentiation of the inner cell mass (ICM) and blastocoele development. The embryo genome becomes activated early in a process known as the maternal to embryo transition, which occurs at a different cell stage in various species. In this transition, cell
regulation is transferred from the maternal RNA to the embryo for continued development.

**Human In Vitro Fertilization (IVF)**

In 1978, Steptoe and Edwards were first to successfully produce a human baby by in vitro fertilization (Steptoe and Edwards, 1978). Since the early 1980s, many of the applied human embryo culture protocols and resulting implantation rates remained relatively unchanged for decades.

Behr and Wang (2004) reported that for the past two decades the majority of human embryos conceived through IVF have been transferred between day 1 and 3 post-insemination at either the pronucleate stage or the cleavage stage. The reason for transferring this stage embryo stems primarily from the inability of the embryo culture systems to support the development of viable blastocysts at acceptable rates. However, reports from a number of investigators, such as; Barnes et al. (1995), Gardner et al. (1998), Jones and Trounson (1999), Cruz et al. (1999), Behr et al. (1999), Schoolcraft et al. (1999), Milki et al. (2000), Gardner et al. (2000) and Alper et al. (2001) have reported increased blastocyst formation and pregnancy results using in vitro culture to the blastocyst stage. Newer culture systems are now more consistent and more day-5 and day-6 blastocysts are available for transfer in women.

There are advantages of culturing embryos to the blastocyst stage, which include being able to select viable embryos for transfer and improving the synchrony of the uterus with embryo development. However, there are also disadvantages of culturing embryos to the blastocyst stage. Extended time in culture is thought to create detrimental effects on human embryos that may, in part, be responsible for the increasing risk of monozygotic twinning (Behr et al., 2000; Menezo and Sakkas, 2002) or possibly a propensity to skew the sex ratio by transferring blastocysts (Menezo et al., 1999). Therefore, a decision must be made on what stage is better to transfer the human embryo. Less time in culture will result in a lower possibility of selecting the highest quality embryo. However, an extended time in culture to the blastocyst stage may cause genetic abnormalities in the embryos (Behr and Wang, 2004).

In the studies reported by Gardner et al. (1998), Cruz et al. (1999), Schoolcraft et al. (1999) and Milki et al. (2000), it has been confirmed that higher implantation rates can be achieved by the transfer of human embryos at the blastocyst stage. It should be
noted that acceptable implantation rates can be achieved with cleavage stage embryos. However, with improved implantation rates with transferred blastocysts, the question of which stage embryo to transfer arises. Alper et al. (2001) indicated contradicting views by citing that a number of reports to date have employed blastocyst transfer in good prognosis patients only (Gardner et al., 1998; Schoolcraft et al., 1999) and although pregnancy rate and implantation rate were very impressive, an excellent success rate with the transfer of cleavage embryos would be expected in this selected group of patients as well.

Extended culture of the embryo usually results in one or two blastocysts of the group with the highest developmental potential at the time of transfer. Furthermore, transfer of a blastocyst stage embryo into the uterus allows for better synchronization of the embryo with the uterus. In the study by Gardner et al. (2000) these assumptions are supported by pregnancy rates of 87% obtained using sequential media in patients who had a good response to gonadotropin stimulation. Comparable results have yet to be equaled with day-3 transfers. Earlier, Cruz et al. (1999) demonstrated blastocyst transfer being effective even for patients with multiple IVF cycle failures. Coskun et al. (2000) and Plachot et al. (2000) have also reported comparable implantation rates for day-3 and for day-5 embryo transfers.

A concerning problem with human Assisted Reproductive Technology (ART) programs is the potential for an increased risk of multiple pregnancies. With the increasing accomplishments of fertility programs there is an increasing demand for high success pregnancy rates among patients. To enhance the chances for a pregnancy, more than one developing embryo is usually transferred into the patient (in the United States not in Europe). The problem with transferring more than one embryo is that multiple implantations occur and these types of pregnancies create greater risk factors to the mother and the IVF offspring. Bergh et al. (1999) indicated concerns regarding the risk of the consequences that multiple pregnancies pose on the mother and children. In a previous study, Martin and Welch (1998) found that women undergoing IVF treatment face a 20-fold increased risk of twins and 400-fold increased risk of higher order pregnancies. Therefore, researchers are faced with the challenge of identifying the best approach to embryo culture and transfer that would result in a pregnancy, but with a minimal chance of a multiple pregnancy. Blastocyst culture appears to be a possible
solution to this problem by limiting the number of embryos transferred into the uterus. Hu et al. (1998) reported that in women who were aged <35, transferring either four poor grade, two fair grade or two good embryos resulted in 35% pregnancy rates with no high order multiple gestation. However, Alper et al. (2001) indicated that when more than two blastocysts are transferred, high order multiple gestations are more likely to happen and the benefits of the technique are lost. Milki et al. (2000) compared transferring 2.4 blastocysts with 4.3 cleavage stage embryos and found a higher pregnancy rate with blastocyst transfer (68% versus 46%) but also a higher triplet rate (12% versus 4%).

Extended culture could possibly have a negative effect on in vitro development and implantation potential where culture conditions are suboptimal. In vitro culture has been reported to affect gene expression and embryo metabolism in animal model systems such as; sheep (Young et al., 2001), bovine (Behr and Wang, 2004) and mice (Doherty et al., 2000), especially with extended culture. Rienzi et al. (2002), Utsunomiya et al. (2002) and Levron et al. (2002) have confirmed these findings. Rienzi et al. (2002) and Utsunomiya et al. (2002) have found no difference between day-3 embryo transfers and day-5 embryo transfers, whereas Levron et al. (2002) showed lower pregnancy rates for day-5 compared with day-3 embryo transfers. It should not be overlooked that effects of extended culture on human embryos may, in part, be responsible for the increased risk of monozygotic twinning (Behr et al., 2000) and increased proportion of male offspring after blastocyst transfer (Menezo et al., 1999).

Furthermore, Leese et al. (1998) reported that embryo culture conditions are known to affect several parameters including the kinetics of development, cell allocation to the inner cell mass and trophectoderm, embryo metabolism and genomic expression. Nagy et al. (1993), Moore and Reik (1996), Dean et al. (1998) and Young et al. (2001) all reported studies showing the effects that culture conditions can have on the expression of imprinted genes on such animals as sheep and mice. The effect of culture conditions on human imprinted genes is still unknown. Young et al. (2001) reported the decreased expression of the IGF2 and imprinting gene H19 on fetuses derived from blastocyst transfer after IVF in sheep. Dean et al. (1998) and Young et al. (2001) proposed that fetal abnormalities found as a consequence of culture during embryo development could, in large part, be due to the change in status of imprinted genes. Beechey (2000) and Khosla et al. (2001) further supported this idea by stating that several imprinted genes
are known to play key roles in murine fetal growth and development. Behr and Wang (2004) have suggested that embryo culture to the blastocyst stage before embryo transfer in sheep and cattle can result in a higher incidence of fetal and perinatal loss. Young et al. (2001) have proposed that fetal abnormalities can be attributed to embryo culture conditions, and in sheep may be linked to reduced expression of the imprinted \(Igf2r\) gene. Doherty et al. (2000) found that H19 expression was affected by different blastocyst culture media using a mouse model. These findings suggested that culture conditions do indeed have an effect on imprinting genes that can be altered by the embryo culture medium.

At this stage it is unknown whether the culture conditions used in human ART programs do affect the expression of human imprinted genes. However, Behr and Wang (2004) have proposed that differences do exist between in vivo-derived and in vitro-derived embryos, possibly attributed to culture induced stress. Kovacic et al. (2002) indicated because of the contradictory outcomes of extended culture, certain doubts still remain about human blastocyst transfer. A number of questions arise when transferring embryos at this stage such as, whether the culture media totally satisfies the nutritional needs of embryos, whether more blastocysts would develop in vivo rather than in vitro, whether prolonged culture has any effect on the developmental potential of the embryo, and whether 5 day cultivation is suitable and justifiable in all cases, even in cycles with a low number of oocytes (Kovacic et al., 2002).

Trounson (1982) reported that in human in vitro fertilization systems the first cleavage should occur between 22 and 30 hours after insemination and thereafter at 10- to 12-hour intervals. He noted that fragmentation and uneven cleavage is indicative of abnormal development or suboptimal culture conditions. The same clinic documented that all of their pregnancies developed from embryos with normal cleavage states, although some had small cytoplasmic fragments in association with equal sized blastomeres. However, Rijnders and Jansen (1998) pointed out that morphology may not be a good predictor of human blastocyst formation because 50% of the good quality day-3 embryos will become blastocysts but so will 20% of the poor quality embryos. Alper et al. (2001) stated since the uterus is a better environment than current blastocyst culture media, we should be cautious in assuming that failure to reach the blastocyst stage indicates that the embryo never had implantation potential. They also noted that
between 2% and 40% of patients will have to cancel their transfers because none of their embryos will develop to day 5 of culture.

Behr and Wang (2004) described the conservative approach their clinic used in switching to the introduction of blastocyst culture. Instead of just switching directly over from day-3 transfers to day-5 transfers they stayed with their normal protocol. Day-3 embryos were transferred and the supernumerary embryos were cultured for 2 or 3 days further. The embryos that developed to day-6 blastocysts were cryopreserved. This ensured that the culture system employed was able to support blastocyst development without compromising day-3 pregnancy rates, and that a suitable cryopreservation system was in place for when the move to fresh blastocyst transfer was accomplished.

Culture System Optimization

An optimal culture system must be in place to achieve the best embryonic development in vitro. Many people find it unethical to perform research on human embryos and it is illegal in most states so an animal model is utilized to form a human IVF culture protocol. Ackerman et al. (1984), Saito et al. (1984) and Dandekar and Quigley (1984) reported the in vitro development of mouse embryos is the most commonly used animal model for quality control of culture media and toxicity testing of materials used for human IVF. Davidson et al. (1988a) agreed that this method allows for the detection of factors that may adversely affect the growth of human embryos in vitro and offers an opportunity to determine optimal culture conditions. Behr and Wang (2004) listed important components of a culture system that included types of incubation chambers, O₂ levels, oil versus open culture, volume of medium, embryonic density and time of gamete co-incubation during the insemination period.

The regulation of temperature, humidity and gas phase is an important aspect in culturing IVF-derived embryos. Trounson (1982) reported the ideal temperature to maintain human oocytes and embryos in culture is 37°C to 37.5°C. Also, short periods of handling oocytes and embryos at 21°C to 25°C appear to have no effect on their cleavage rates. In an earlier study, Trounson et al. (1976) showed that reducing temperatures to 4°C proved to be deleterious to pig embryos and early cleavage stage cattle embryos. However, when investigators from the same group reduced temperatures to 4°C for a few human embryos to evaluate their sensitivity to cooling they found that embryos continued development in vitro after cooling.
Trounson (1982) has stated that if oil is not used to cover the embryos during culture, then 100% humidity is not maintained, which is necessary to maintain the correct osmolarity of culture medium. A humid chamber was recommended to ensure that the culture medium (with or without oil coverage) does not evaporate when the incubator used is unable to achieve the correct humidity level.

Trounson (1982) also reported that when using bicarbonate-based culture media, a 5% CO₂ gas phase must be maintained to control the pH. Human pregnancy rates were similar after using 5%, 10% and 20% O₂, so the use of 5% O₂ as a standard remains debatable.

Although there have been advances in producing in vitro-derived embryos, most of the commonly used culture techniques have not changed, resulting in unchanged implantation rates since the 1980s. The majority of human embryos produced through IVF have been transferred between days 1 and 3 because past culture systems were unable to support adequate embryo development to the blastocyst stage. As new culture systems are incorporated into the human IVF clinics, more day-5 and day-6 human blastocysts are now available for transfer.

Once an acceptable culture system is in place, there still remains the possibility of poor blastocyst development due to endotoxins in the medium. Fishel et al. (1988) defined these endotoxins as complex phospholipid-polysaccharide-protein macromolecules released after the death of bacterial cells. These endotoxins are associated with gram-negative bacteria and can be found in the cell wall of the bacteria. These endotoxins are released after either natural autolysis or artificial disruption. One of the most common types of endotoxins is found in the form of saprophytic gram-negative bacilli that grow in the water used for culture media. The problem is these endotoxins are heat stable and most often are not destroyed by autoclaving.

Jones et al. (1982) commented on the improved pregnancy rate in their human IVF-ET program after more stringent quality control was applied in their laboratory procedures. Similarly, Fishel et al. (1988) emphasized that maintaining high quality control in a human IVF laboratory was essential for consistent success. Previously, Fishel and Jackson (1986) described many of the problems associated with the culture of human embryos in vitro. After noticing fluctuations in the in vitro culture results of their own clinical IVF cycles over a period of time, Fishel et al. (1988) conducted a thorough
investigation and found the culture medium as a source of contamination. They detected endotoxins in the culture medium. The problem appeared when an increased number of cytoplasmic fragmentation began to show up in the embryo. To explore the cause of these abnormalities batches of culture medium were tested proceeding, during and immediately after use for the presence of endotoxins.

In addition, Fishel et al. (1988) tested manufacturer-supplied 1X and 10X concentrates of Earle’s culture medium in liquid form, homemade Earle’s culture medium, the on-site Milli Q (water purified to between 17 and 18 MQ) and reverse osmosis water (Millipore). Paraffin source, pyruvate and penicillin were also tested for endotoxins. They demonstrated that concentration of endotoxins at >1 ng/ml significantly reduced the overall number of oocytes fertilized, but there was no observable effect if the level of endotoxin was <1 ng/ml. The most surprising finding was the level of endotoxins in the culture medium supplied by a major culture media manufacturer. The 1X culture medium had an endotoxin level >0.5 ng/ml and the 10X culture medium had a level of 1 ng/ml. Tap water that had passed through the Millipore reverse osmosis system and was stored in a 90-liter tank also was positive to 0.1 ng/ml, but further purification through the deionizers and ultrafiltration unit before use reduced this to undetectable amounts.

Researchers have reported abnormalities in developing embryos due to the presence of endotoxins in the culture medium. Silver (1981) investigated the effect of Escherichia coli and demonstrated irreversible damage to cells within 10 minutes of exposure to endotoxin. Fishel et al. (1988) have reported that the human embryo results in increased fragmentation, along with irregular membrane patterns and granular cytoplasm in the presence of endotoxin. Silver (1981) and Snyman and Van der Merwe (1986) have shown endotoxins damaging internal cell organelles, such as endoplasmic reticulum, the cristae of the mitochondria, detachment of ribosomes, granulation of the nucleus and leakage of the cytoplasm. In contrast, Fishel et al. (1988) suggested that endotoxins do not affect fertilization rate. Reduced incidence of fertilization did not occur in endotoxin-positive medium as compared with endotoxin-negative medium, and cleavage occurred at a similar rate in both media after fertilization. It was concluded that endotoxin contamination may be more obvious from the irregularity and appearance of blastomeres and the degree of cytoplasmic fragments.
Bovine In Vitro Fertilization (IVF)

Many of the same requirements that are essential in the survival and transfer of human embryos are necessary for the culture and transfer of bovine embryos. However, the reasons for bovine IVF procedures vary from those of humans, whose problems are mainly caused by infertility. First and Parish (1987) reported that the reasons for developing systems for fertilization and development of bovine embryos in vitro were for the production of a large number of genetically superior offspring and the production of transgenic animals.

Similar to human IVF culture, First and Parish (1987) emphasized that for ruminants, sperm concentration, time of sperm-ovum interaction, medium utilized and temperature play a role in successful fertilization, resulting in developmentally competent embryos. One of the main reasons for failure of fertilization occurring in an in vitro system is the occurrence of polyspermy. In vivo, oocytes encounter a much smaller number of sperm at the time of fertilization and have more time to express blocks to polyspermy. The time that sperm and oocytes are allowed to interact in vitro becomes important because early blocks to polyspermy in mammalian oocytes may not occur as rapidly as in invertebrates (see reviews by Wolf, 1981; Shapiro, 1981) and the block to polyspermy is reduced in aged oocytes (Hunter, 1967). Furthermore, Leibfried-Rutledge et al. (1987) reported in vitro matured oocytes may not develop the block to polyspermy to the same effectiveness as in vivo matured oocytes. Therefore, sperm concentrations and time of sperm-oocyte interaction should both be controlled to minimize polyspermy (First and Parrish, 1987).

Medium composition and temperature can effect maturation and culture, as well as influence fertilization success. Parrish et al. (1985) and Susko-Parrish et al. (1985) have shown that glucose blocks bovine sperm capacitation. First and Parrish (1987) reported in vivo fertilization occurs at core body temperature, which is 38°C to 39°C for cattle, 39°C for sheep and 38°C to 39°C for goats. In cattle, Lenz et al. (1983) have reported in vitro fertilization frequencies are very dependent on temperature, and highest frequencies occur at 39°C. First and Parrish (1987) suggested that temperature not only controls efficiency of capacitation but also the ability to undergo a physiological acrosome reaction in response to interaction with the zona pellucida.
In early studies, Thibault (1966) reported that the culture of in vitro-produced bovine embryos led to their arrest at the 8- to 16-cell stage. Over the years this observation has been explained as the inability of in vitro conditions to allow expression of the embryonic genome, since the bovine embryo has been found to be transcriptionally activated near this time (Barnes and Eyestone, 1990), with resulting changes in patterns of protein formation (Frei et al., 1989). Rexroad (1989) and Bongso et al. (1990) reported various somatic cells could be used in embryo co-cultures and in conditioned medium (Eyestone and First, 1989) to overcome arrested development in vitro. Rexroad (1989) and Eyestone and First (1989) indicated that the positive impact of somatic cell co-cultures on bovine embryo development was attributed to transfer of embryotrophic factors and/or reduction of embryotoxic factors in the medium.

There are a variety of methods used for culturing bovine embryos. These include the co-culture system with somatic cells, such as oviduct epithelial cells (Eyestone and First, 1989; Ellington et al., 1990; Aoyagi et al., 1990; Fukui, 1990; Kim et al., 1990; Saeki et al., 1990), uterine fibroblasts (Voelkel et al., 1985; Ellington et al., 1990), cumulus cells (Goto et al., 1988; Aoyagi et al., 1990; Fukuda et al., 1990; Younis and Brackett, 1991) and trophoblastic vesicles (Camous et al., 1984; Heyman et al., 1987; Pool et al., 1988; Aoyagi et al., 1990). However, Bavister et al. (1992) pointed out that under these culture conditions the portion of inseminated oocytes that develop to the morula or blastocyst stage ranges from 25% to 40%.

It should not be overlooked that Bavister et al. (1992) stressed that co-culture systems using somatic cells was a complex culture medium, which usually contains blood serum, that makes it difficult to analyze the factors involved in embryo development. Keskintepe et al. (1995) indicated that to understand the requirements for oocytes going through maturation, the fertilization process and then to uterine-stage embryos, all unknowns should be eliminated from supporting chemical and physical culture conditions.

Over the years there have been difficulties developing IVF procedures for farm animals. The first IVF calf was produced in 1982 (Brackett et al., 1982) followed by the production of goat (Hanada, 1985), sheep (Cheng et al., 1986), pig (Cheng et al., 1986) and horse (Palmer et al., 1991) IVF-derived offspring. First and Parish (1987) were among the first to compile data indicating that the efficiency of producing in vitro offspring
was less than that achieved by natural mating. Their results showed that ~10% of the bovine oocytes produced IVF-derived offspring. They also indicated that the most limiting step in the process was the inefficiency of development of embryos in vitro from the pronuclear to the blastocyst stage. For immature bovine oocytes recovered from small follicles (1 to 5 mm in diameter) an additional limitation was the failure to acquire competence for development to the pronuclear stage (Leibfried-Rutledge et al., 1987). Staigmiller and Moor (1984) and Critser et al. (1986) concluded that immature oocytes can obtain embryo developmental competence by co-culturing with abundant pre-ovulatory granulosa or cumulus cells, which receive FSH, LH and estradiol priming.

**Defined Culture Systems of In Vitro-Produced Mammalian Embryos**

The energy requirements of bovine embryos during cleavage stages have not been fully defined (Rexroad, 1989; Rieger, 1992), in part, because of the 8- to 16-cell in vitro developmental block (Thibault, 1966; Brackett et al., 1982; Camous et al., 1984; First and Parrish, 1987; Aoyagi et al., 1990; Keskintepe et al., 1995). In addition, media commonly used for bovine embryo culture are complex, usually containing up to 20% serum (Wright and Bondioli, 1981). Fukui et al. (1991), Pinyopummintr and Bavister (1991), Takahashi and First (1993), Shamsuddin et al. (1994) and Goto et al. (1994) have eliminated somatic cells as components of the embryo culture system; however, they did not replace the bovine serum or bovine serum albumin (BSA) in maturation, fertilization or the culture medium, and these components contain unknown factors that contribute beneficial effects on the embryos (Brackett, 1981). Larson et al. (1992) and Goto et al. (1994) have reported improved viability of uterine-stage bovine embryos produced in vitro in co-culture-free medium by achieving similar successful embryo transfer rates yielding offspring.

The development of defined bovine embryo culture systems holds promise for ongoing efforts in reproductive physiology and for utility in the embryo transfer industry (Keskintepe and Brackett, 1996). Although a variety of embryo culture systems are used for in vitro embryo production adequate definition to assure quality control and repeatability is still lacking. Eliminating culture medium variability would lead to better understanding of embryo development and thus, over the years attempts have been
made to simplify (Pinyopummintr and Bavister, 1991; Kim et al., 1993; Brackett and Keskintepe, 1994; Rosenkrans and First, 1994; Goto et al., 1994, Keskintepe et al., 1995; Eckert and Niemann, 1995).

When evaluating new culture systems, it is ideal to be able to perform multiple tests on the same batch of culture medium. However, IVF clinic regulations (Gerrity et al., 1992; McLendon, 1992) and proposed recommendations (Purdy, 1982; Quinn et al., 1984) on the amount of time embryo culture media can be stored have been implemented over the years of a shelf life of no longer than 2 weeks at 4°C for human IVF culture. Researchers have disagreed with this shelf life time restraint and have suggested using refrigerator-stored or frozen-stored culture media to increase the opportunity for more tests to be conducted on a single batch of medium (Naz et al., 1986; Bernart et al., 1990; De Silva, 1993; Bell et al., 1995). The advantages of media stored for extended periods of time include reduced variability by using one tested batch of medium, more efficient and less costly embryo production, availability of cross-testing and increased number of assays performed could facilitate a more defined human IVF culture system.

Using laboratory animals, Bavister et al. (1992), Trounson (1992) and Gardner (1994) have helped define in vitro conditions and increased the understanding of molecular mechanisms that promote or impede early embryo development. Amino acids (Rosenkrans et al., 1989; Zhang and Armstrong, 1990; Kim et al., 1993; Rosenkrans and First, 1994; Keskintepe et al., 1995; Miyoshi et al., 1995), energy substrates (Takahashi and First, 1992; Rosenkrans et al., 1993;), citrate (Gray et al., 1992; Keskintepe et al., 1995) and vitamins (Kane and Bavister, 1988a, 1988b; Takahashi and First, 1992; Rosenkrans and First, 1994), to name a select few, have been shown to profoundly influence embryonic development in vitro.

**Amino Acids**

Amino acids play an important role in mammalian IVF culture as energy substrates, osmolytes, regulators of pH and enzyme activity, scavengers of free radicals, chelators of heavy metals, precursors of macromolecules such as proteins and nucleic acids and mediators of transport across the cell membrane (Bavister, 1995; Liu and Foote, 1995; Lee and Fukui, 1996). However, the beneficial effects can be restricted to
certain amino acids (Bavister, 1995; Steeves and Gardner, 1999) and may be affected by the base medium (Van Winkle and Campione, 1996).

Kane et al. (1986) reported that development of hamster embryos in vitro is enhanced by the addition of amino acids and vitamins. In a classic study, Carney and Bavister (1987) found that development of hamster embryos was increased when 20 amino acids were added to Tyrode’s albumin-lactate-pyruvate (TALP) medium. Furthermore, they noted that the addition of glutamine alone to be as good as or better than the addition of the 20 amino acids or various combinations of amino acids. Meyen et al. (1989) reported the opposite results with pig embryos, stating that these embryos may have retarded in vitro development in the presence of minimum essential medium (MEM) or basal medium Eagle (BME) with amino acids. However, Stone et al. (1984), Rosenkrans et al. (1989) and Hagen et al. (1991) reported normal development of pig embryos with free amino acids in the culture medium. With bovine embryos, Takahashi and First (1992) reported that development improved when both BME essential and MEM nonessential amino acids were added to a modified synthetic oviduct fluid medium (mSOF).

Rosenkrans et al. (1989) evaluated the effects of glutamine, methionine, phenylalanine and isoleucine on pig embryo development. The reason these particular amino acids were chosen for this study is because Bavister et al. (1983) noted improvement in hamster embryo development in vitro when these amino acids were added to the culture medium. Gwatkin and Haidri (1973) had previously reported that of the 13 amino acids in MEM, glutamine, methionine, phenylalanine and isoleucine were the most critical for in vitro maturation of hamster oocytes. In his study, Rosenkrans et al. (1989) added these four amino acids with the presence of serum and with the absence of serum in the culture medium. The results indicated that in the presence of 10% lamb serum the addition of phenylalanine, methionine and isoleucine significantly improved blastocyst expansion, but when lamb serum was omitted, addition of the same three amino acids was detrimental to embryo development. It was found that glutamine was detrimental to pig embryos in the presence of serum but beneficial when the culture medium did not contain serum. Methionine was beneficial to pig embryos in the absence of serum and was the only factor that increased the number of cell nuclei per embryo.
These porcine embryo findings suggested that it is obvious that other factors are required by pig embryos that were not evaluated in this experiment (Rosenkrans et al., 1989). Thus serum likely contains those unknown components, including nutrients and growth factors. However, even in the presence of serum there is still a difference in the development of in vitro-produced compared with in vivo-produced pig embryos. Similar retardation patterns of in vitro-produced embryos have been recognized for the embryos of other animal species (Bavister, 1987). Until researchers are able to fully understand and duplicate the exact conditions provided by the reproductive tract of mammals during early pregnancy these developmental inhibitions will remain present.

It has been proposed over the years that embryo requirements for amino acids change according to the developmental stage. Kim et al. (1993) found almost no development of bovine 1-cell embryos to the blastocyst stage when mTLP-PVA without amino acids was used, whereas, 9% to 13% of the embryos developed when the medium (glucose-free) was supplemented with 18 amino acids (all contained in HECM) and with one extra amino acid (L-tyrosine) but without taurine and glutamine, at low concentrations (0.02 - 0.2 mM). Takahashi and First (1992) also reported that supplementation of 20 amino acids to a chemically semi-defined medium with BSA markedly improved development of bovine 1-cell embryos to the morula stage. Kim et al. (1993) noted these findings indicate the importance of amino acids for in vitro development of bovine embryos. It was previously shown that the uptake of amino acids increases from the 8-cell to the blastocyst stage in mouse embryos (Brinster, 1971). In addition, amino acids have also been found to be a requirement for in vitro development of hamster (Bavister et al., 1983; Carney and Bavister, 1987; Kane and Bavister, 1988b), rabbit (Daniel and Olson, 1968; Kane and Foote, 1970), rat (Zhang and Armstrong, 1990), pig (Rosenkrans et al., 1989) and sheep (Zhang and Armstrong, 1990) embryos.

To further support the idea that amino acid needs are different at different stages of embryo development, Schini and Bavister (1988) and Seshagiri and Bavister (1991) showed that with the chemically defined medium HECM containing 20 amino acids, development of hamster 2-cell and 8-cell embryos to the morula and blastocyst stages can be obtained. However, Bavister and Arlotto (1990) reported that hamster 1-cell embryos are inhibited from development past the 4-cell stage in this medium, showing that a group of amino acids inhibit the development in vitro.
Rosenkrans and First (1994) evaluated the effect of free amino acid and vitamin supplementation to developing bovine embryos cultured in CR1aa medium. A claim was made that this was the first report demonstrating an additive effect of the fibroblast essential amino acids (EAA) and nonessential amino acids (NEA) on embryo development. The results of the study concluded that the addition of BME and MEM amino acids to CR1 increased the developmental rate of bovine zygotes in vitro. This 1994 paper designated the use of CR1 medium with EAA and NEA as CR1aa. In a previous paper, Monson et al. (1992) reported that bovine embryos developed in the basic CR1 medium established pregnancies as well or better than embryos developed in co-culture with oviductal cells after transfer to recipient cows. Betteridge and Flechon (1988) suggested that the increased morula to blastocyst ratio with the addition of amino acids to the culture medium was similar to that of in vivo production. Takahashi and First (1992) reported that the addition of both EAA and NEA to mSOF increased the cell number of bovine IVF blastocysts.

Rosenkrans and First (1994) proposed that amino acids were likely acting as energy substrates, pH regulators and/or as a pool for de novo protein synthesis. This study showed the amino acid composition of NEA and EAA with glutamine (1mM) increased the development of bovine embryos in vitro regardless of the mechanism(s) involved by the amino acid(s).

**Glucose and Phosphate**

Pinyopummintr and Bavister (1991), using a chemically defined medium (HECM) containing 20 amino acids but not pyruvate, protein, glucose and phosphate, reported that blastocyst development of bovine embryos was depressed in the presence of glucose and phosphate when compared with phosphate alone. They reported 13.8% of embryos in TCM-199 containing both glucose and phosphate, the same percentage of embryos (14.3%) as that for HECM with phosphate developed to the blastocyst stage in the same experiment. Kim et al. (1993) indicated this inconsistency makes it difficult to explain the exact role(s) of glucose and phosphate in chemically defined media. Bovine blastocyst development was greatly inhibited in the presence of glucose, regardless of the presence of phosphate, in a simple, chemically defined, protein-free medium (mTLP-PVA) with 19 amino acids, lactate and pyruvate. Schini and Bavister (1988) obtained
contrasting results using hamster 2-cell embryos cultured in chemically defined medium (HECM).

In both species (hamster and cattle) culture inhibition from glucose did not occur in the absence of phosphate (Schini and Bavister, 1988; Pinyopummintr and Bavister, 1991). However, in a chemically semi-defined medium with phosphate, the deleterious effect of glucose (when present throughout culture) has been reported on bovine (Takahashi and First, 1992) and ovine (Thompson et al., 1992) embryo development to the morula or blastocyst stage. In contrast, Javed and Wright (1991) and Rieger et al. (1992) indicated that the embryonic metabolism of glucose markedly increased in the bovine embryo from the 2-cell stage to the blastocyst in bovine embryos. However, Rieger and Loskutoff (1994) reported that cumulus-enclosed bovine oocytes utilize glucose and pyruvate during the first 18 hours of their in vitro maturation in preference to glutamine, and glutamine metabolism is amplified 18 hours after the beginning of maturation.

Takahashi and First (1992) and Wang et al. (1990) have reported that when glucose was added in various concentrations to synthetic oviduct fluid or conditioned medium, bovine embryo development decreased as the concentration of glucose increased. Rosenkrans et al. (1993) found that the development of bovine embryos was inhibited when glucose was added to the CR2 medium. CR2 medium consisted of sodium chloride (109.5 mM), potassium chloride (3.1 mM), calcium chloride (2.5 mM), magnesium sulfate (0.5 mM), sodium bicarbonate (26.2 mM) and EDTA (0.01 mM) with 3 mg fatty acid-free BSA per ml. This inhibition of development occurred even in the absence of exogenous phosphate. These data contradict the results of others (Schini and Bavister, 1988; Pinyopummintr and Bavister, 1991) indicating that the inhibitory effect of glucose on bovine and hamster embryo development occurred only in the presence of phosphate. However, it should be noted that the composition of the base medium in those studies was different from that of Rosenkrans et al. (1993).

**Lactate and Pyruvate**

Kim et al. (1993) reported that when lactate (10 mM) and pyruvate (0.5 mM) were omitted from mTLP-PVA containing 0.35 mM phosphate and amino acids, the first cleavage of bovine 1-cell embryos was completely inhibited in the chemically defined condition used. However, when supplementing the two substrates alone, each could
equally support the development of bovine 1-cell embryos. The individual substrates not only supported these bovine embryos to the 2-cell stage but also to the blastocyst stage as well. Although McKiernan et al. (1991) reported that pyruvate was inhibitory to the development of hamster 1-cell embryos, previous investigators have reported no such inhibitory effect on development of bovine (Takahashi and First, 1992) and ovine (Thompson et al., 1992) 1-cell or 2-cell embryos to the morula or the blastocyst stage in chemically semi-defined media with serum albumin.

The energy requirements of murine embryos have been studied extensively (Quinn and Wales, 1973; Cross and Brinster, 1973; Chatot et al., 1989; Lawitts and Biggers, 1991a); however, few reports have been published on energy metabolism by embryos from domestic farm animals (Rieger et al., 1992). Rosenkrans et al. (1993) evaluated the pyruvate and lactate requirements of bovine embryos in vitro. Rosenkrans et al. (1993) reported that in a simple medium (serum-free) the addition of pyruvate does not appear to be necessary for in vitro development of bovine embryos when L-lactate was present. They also reported that when pyruvate was added as the only energy substrate, concentrations greater than 1 mM decreased the percentage of embryos developing to the blastocyst stage. These results are in agreement with those of Cross and Brinster (1973) when they cultured mouse zygotes. Cross and Brinster (1973) suggested that bovine embryos appear to be more tolerant of increased concentrations of L-lactate than of pyruvate, but less tolerant of lactate concentration than murine embryos. Rosenkrans et al. (1993) indicated that the use of L-lactate in preference to pyruvate is associated with the conversion of L-lactate to pyruvate by lactate dehydrogenase (LDH). Results reported by Johnson et al. (1991) suggested that LDH could be used as an indicator of bovine embryo viability.

Chatot et al. (1989) and Cross and Brinster (1973) indicated that a lactate:pyruvate ratio approaching 120 was optimal for in vitro development of murine embryos. For bovine embryos, the results of Rosenkrans et al. (1993) suggested that the lactate:pyruvate ratio was less important than the total lactate or pyruvate concentration. The primary controller appears to be the lactate concentration when both lactate and pyruvate are added to the medium.
Vitamins

There is very little information available to date on the role of vitamins for embryo development, but it is probable that their role in embryos is generally similar to their role in mammalian cells (Kane and Bavister, 1988a). Hawthorne (1982) and Vance (1986) reported inositol is essential for the synthesis of certain membrane phospholipids in cells and it is possible that at the blastocyst stage it becomes necessary for the synthesis of phospholipid components of new membranes.

Kane et al. (1986) showed that the addition of amino acids and a group of 11 water-soluble vitamins and growth factors from Ham’s F-10 medium to a simple medium containing bovine serum albumin stimulated development of 8-cell and morula stage hamster embryos to hatching blastocysts in vitro, but hatching was delayed when compared with the in vivo development. In a subsequent study, Kane and Bavister (1988a) examined which of 11 water soluble vitamins stimulated hamster embryonic development at the blastocyst stage. This question is of particular interest because there is only one other mammalian species (the rabbit; Kane, 1988) for which this information is available. Of the 11 vitamins only two showed the effects of successive addition to the culture medium, which was a modified Tyrode’s solution (TLP-PVA, Bavister et al., 1983; Boatman, 1987) without phenol red supplemented with amino acids. The two vitamins that showed a difference in development with their addition to the culture medium were inositol and choline.

Frozen Storage of In Vitro Culture Media

The quality of the culture medium is a crucial factor influencing the success of IVF/IVC human oocytes and embryos in a program for infertility treatment (Jones et al., 1982; Purdy, 1982; Trounson, 1982; Dandekar and Quigley, 1984; Quinn et al., 1984; McDowell et al., 1988). Therefore, Purdy (1982), Trounson (1982) Vijayakumar et al. (1987), Naaktgeboren (1987), Davidson et al. (1988a, 1988b) and McDowell et al. (1988) have recommended the performance of an assay for mammalian embryo culture to evaluate the suitability of the medium before using it for human IVF. Purdy (1982) and Quinn et al. (1984) proposed that a preparation of culture medium stored at 4°C for longer than 1 to 2 weeks should not be used for human IVF. Also, guidelines have been
set by the American Fertility Society (AFS) for ART programs (Gerrity et al., 1992) and the College of American Pathologists (CAP) (McLendon, 1992) to ensure clinics adhere to this storage time limit. It takes 4 to 5 days for the results of the embryo culture assay to be evaluated in all of the available test models. Therefore, only a few days are left after the test for the medium to be used. Due to the limited storage period (shelf life), no time is available to repeat an assay yielding unclear results or to cross-test with other IVF laboratories.

The maintenance of a high quality culture system for human IVF is a difficult task for laboratory technicians. The quality of the chemicals and the water used for media preparation can vary from batch to batch and influence the outcome of human IVF procedures (Codon-Mahony et al., 1985; Vijayakumar et al., 1987; Yovich et al., 1988). Even minimal contamination of the medium, for example with endotoxins (Snyman and Van der Merwe, 1986; Fishel et al., 1988) or with surgical glove coating (Naz et al., 1986) has been reported to reduce the pregnancy rates after embryo transfer.

Bernart et al. (1990) proposed that fertilization of the oocyte and the first 2 or 3 cleavage steps are almost uninfluenced by subtle changes in culture medium quality. Leung et al. (1984), Grillo et al. (1987) and Fishel et al. (1988) indicated that the “morphological quality” of the embryos at the time of the embryo transfer (usually in the 4-cell stage) gives very little information relating to the chance of embryo implantation. Bernart et al. (1990) reported that subtle damage to embryos often does not occur before the human morula or blastocyst stage, 2 to 3 days after the day of embryo transfer.

In a pilot study, Curole et al. (1988) placed “spare” human embryos in culture up to the blastocyst stage. This procedure provided information related to the quality of the culture system, but such an “assay” performed during routine IVF work can obviously caused ethical and/or legal concerns. Quinn et al. (1984) demonstrated how essential it was for an IVF clinic to have a reliable quality control system in an animal model. Since some test models fail to detect subtle changes in medium quality several authors (Davidson et al., 1988a, 1988b; Fleetham and Mahadevan, 1988; Rinehart and Bavister, 1988) reported it would be very useful to have the media cross-tested between different IVF laboratories before using them on human IVF. With the recommended storage period at 4°C of 1 to 2 weeks, there is little time available for these extensive tests (Purdy, 1982; Quinn et al., 1984; McLendon, 1992; Gerrity et al., 1992).
With such a limited storage time for the use of culture media, IVF directors are faced with complications that accompany this restraint. Preparing culture medium every 2 weeks can be time consuming and expensive, but viewed as necessary to operate a successful IVF program. It has been suggested that this 2-week storage limitation is unnecessary and no developmental decrease is seen with in vitro-produced embryos cultured in media stored for longer periods of time (Naz et al., 1986; Bernart et al., 1990; De Silva, 1993; Bell et al., 1995). Naz et al. (1986) showed that storing Ham’s F-10 medium for up to 425 days at 4°C did not affect 2-cell mouse embryo development to blastocyst culture. However, the 2-cell mouse embryo may not be sensitive to subtle changes in the culture medium and, therefore, may not have detected any differences between the freshly prepared and the stored medium (Silverman et al., 1987; Davidson et al., 1988a).

When freezing culture media it must be taken into account that Na⁺ solutions tend to precipitate after freezing and thawing. In most embryo culture media almost the entire source of osmolarity comes from the Na⁺ concentration. Most of the time, increased osmotic pressure is a direct affect of increased Na⁺ levels. However, extracellular Na⁺ can affect intracellular pH via its effect on the Na⁺/H⁺ anti-porter (Lane and Bavister, 1999). High NaCl concentrations and or osmotic pressure of a medium can affect the development of embryos in vitro. Summers et al. (1995) indicated lower osmotic pressure and a reduction in NaCl concentration were partially responsible for the improved development of mouse embryos in KSOM. Lim et al. (1994) and Liu and Foote (1996) recommended reducing the osmotic pressure of bovine culture media to <270, however, the most widely used human culture media (SOF, TCM-199, CR1) have higher osmotic pressures than this.

Van den Berg and Rose (1959) reported the negative effect of temperature in the freezing zone on pH and composition of potassium and sodium phosphate solutions is a result of salt and ice precipitation. Previous studies by Lovelock (1953a, 1953b) and Lovelock and Polge (1954) showed that the damage suffered by erythrocytes and spermatozoa was related to the sodium chloride concentration reached in the suspension during freezing, causing increased attention to be focused on the effect of salts during freezing. As sited by Van den Berg and Rose (1959), Finn (1932) showed that the pH of the liquid portion of frozen muscle juice decreased with temperature below
the freezing point and that denaturation of muscle juice proteins reached a maximum at
temperatures when the pH was below 6.0 with a salt concentration in the solution of
around 0.8 M. Previously, Harvey (1918) found that the pH of cabbage juice decreased
during freezing (see Van den Berg and Rose, 1959). Subsequently, Tessier and Rose
(1956) noted that the pH of milk ultrafiltrate changed from 6.7 to 5.8 during freezing (see
Van den Berg and Rose, 1959). They suggested that such a pH change could decrease
the stability of the casein of frozen milk. Rey (1957) stated that when temperature is
lowered in addition to an increase in salt concentration, pH changes may occur and
affect the material being frozen. Van den Berg and Rose (1959) indicated that since the
pH of solutions depends on composition and precipitation of either ice or salt, then
freezing should affect the pH.

Van den Berg and Rose (1959) showed that substantial changes in pH and
composition occur in buffer solutions of sodium and potassium phosphates during
freezing, depending on initial pH and relative contents of sodium and potassium. In
another study, Van den Berg (1959) indicated that biological materials contain other salts
in addition to sodium and potassium phosphate, and these also may have appreciable
effects on pH changes during freezing. Sodium and potassium chlorides occur most
frequently among these additional salts and are usually present in relatively large
concentrations. However, Lovelock (1953b), Lovelock and Polge (1954), Harrison
(1956) and Lusena and Rose (1956) have stated that biological material is often frozen in
the presence of sodium chloride.

Van den Berg (1959) also reported the pH and composition of the solutions from
which both sodium and potassium phosphate are precipitating, either with or without a
chloride, as well as the freezing points of these same solutions. Thus, within the pH
range of 3.3 to 7.5, a complete range was given of the changes that may occur in pH,
composition and freezing point of mixed solutions of sodium, potassium, phosphates and
chlorides. Van den Berg (1959) indicated that the addition of either sodium or potassium
chloride to material buffered with phosphates may cause marked changes in pH during
freezing. Lea and Hawke (1952), Harrison (1956) and Lovelock (1957) indicated that
such changes will significantly alter the stability of frozen biological materials. Van den
Berg (1959) suggested that buffer solutions in which biological materials are to be frozen
should, therefore, be chosen so as to minimize these pH changes.
Since the stability of colloid systems depends on pH, as well as, ionic strength of the solution in which they were suspended, pH changes, such as were demonstrated in the phosphate solutions, are likely to affect the stability of various biological systems such as, erythrocytes, spermatozoa and bacteria during freezing (Van den Berg and Rose, 1959). Given that the development of acidity during freezing is harmful, the use of a high potassium buffer may be beneficial.

Van den Berg and Rose (1959) also have indicated that supersaturation may cause only temporary changes in pH. Large degrees of supersaturation for one or more salts occurred frequently and were sometimes maintained for several hours or even days even though freezing mixtures were seeded. They reported these metastable states did not affect the pH and composition of the stable state finally reached, but they represented temporary changes in pH and composition that may be important in the freezing of biological materials.

Lopata et al. (1980) and Purdy (1982) described methods of freezing and storing concentrated stock solutions of culture media for IVF. The disadvantage with these methods when used for culture is the addition of water to the post-thaw medium. Once water is added the pH and osmolarity of the medium must be re-adjusted and another quality test in the animal model has to be performed after the preparation of the “ready-to-use” medium.

Bernart et al. (1990) investigated a very easy method of frozen storage of Ham’s F-10 medium for several months. The results provided “ready-to-use” culture medium for human IVF without having to re-adjust and test the medium after thawing. Two application models were used; an animal IVF model and a human IVF model. In the storage process, separate aliquots of 33 ml of Ham’s F-10 stock solution and 1 ml aliquots of sodium bicarbonate stock solution were frozen at -20°C. They explained the bicarbonate must be frozen and stored separately, because if not components of the medium precipitate out during the freezing–thawing process.

Bernart et al. (1990) cultured 2-cell stage mouse embryos in fresh medium (control) and in medium frozen for 2 weeks, 1 month, 2 months, 4 months and 6 months, respectively. The developing embryos were then evaluated for expanded blastocyst and hatched blastocyst rates. After the test series in the mouse model, two different IVF centers (University Women’s Hospital of Heidelberg and Municipal Women’s Hospital of
Darmdstadt) applied the frozen-thawed medium to human IVF. Bernart et al. (1990) collected data from three subgroups where different periods of frozen storage medium (up to 1 month, 1-2 months, 2-3 months) were evaluated and compared with the results of cycles in which non frozen culture medium was used (storage at 4°C for up for 5 days). In both the animal and human culture models, there was no significant difference between the subgroups cultured in fresh medium compared with medium stored frozen for various time intervals. These results showed that there is no loss of stability of the frozen-stored Ham’s F-10 medium within the tested period of 6 months for the animal model and 3 months for the human model.

The storage of culture media creates advantages that do not exist with reproducing stock medium every 2 weeks. A number of batches can be made at the beginning of each IVF cycle, tested with the 1-cell mouse embryo bioassay and selected for those batches that perform the best. Those batches can then be properly stored in a refrigerator at 4°C and subsequently used for human embryo cultures during that cycle. This would likely minimize variability among culture media and cut down on expenses and technician time. Also, storage would enable gametes and embryos from a group of patients to be exposed to the same batch of culture medium.

De Silva (1993) evaluated the effect of storage of Ham’s F-10 medium on 1-cell mouse embryo development in vitro. Embryos were cultured in one of seven groups; fresh, 1-, 2-, 3-, 4-, 5- or 6-month old medium, with four to six replicates for each month of comparison. The percentage of development of small, expanded/collapsed, hatched, and all (small, expanded/collapsed, and hatched stages combined) blastocysts by 96 and 120 hours after culture was calculated based on the initial number of 2-cells. This study showed there was no significant difference in embryo development to 2-cells, whether embryos were cultured in fresh or stored medium. The results also showed that there was no difference in the development to small, expanded/collapsed, hatched or all blastocysts either at 96 or 120 hours after initiation of the culture. The findings of this study indicated that Ham’s F-10 medium can be stored in a refrigerator at 4°C for longer than the recommended 2-week period without compromising 1-cell mouse embryo development in vitro. These results are in agreement with two earlier studies (Naz et al., 1986; Bernart et al., 1990) showing that Ham’s F-10 medium can be stored refrigerated (4°C) or frozen (-20°C) without affecting 2-cell mouse embryo development in vitro.
Subsequently, Bell et al. (1995) tested the storage ability of refrigerated Ham’s F-10 culture medium in human assisted reproduction programs. The group exposed human gametes and early pre-implantation embryos to a single preparation of Ham’s F-10 medium (2-liters) maintained in refrigerated storage (4°C) for a period of 3 months. The storage life was measured by the ability of the medium to support fertilization and early embryos in vitro. Associated implantation rates were used as a later indirect indicator of the quality of the medium. Patients in the study were separated into two treatment groups, IVF or ZIFT, determined by respective fertility treatment. The oocytes and embryos of the IVF patients or oocytes and fertilized oocytes of the ZIFT patients, were exposed to the same batch of Ham’s F-10 medium stored at 4°C in the dark for periods of up to 1, 2 and 3 months. They compared the results of 2 and 3 month storage periods with 1 month storage periods. The fertilization and clinical pregnancy rates between 1 month storage and 2 and 3 month storage for IVF-ET and ZIFT patients were not significantly different. Based on these results, Bell et al. (1995) suggested that reconstituted Ham’s F-10 medium can be stored at 4°C for at least 3 months, since it still provided adequate support for fertilization and early in vitro development to the human 4-cell stage embryo.

Over the past three decades, in vitro embryo culture and transfer has become increasingly popular among different animal species making it crucial to have an efficient culture system in place allowing for adequate embryo development. In whatever species, researchers agree that good quality control in the laboratory is essential to the success of in vitro fertilization. Most laboratory directors believe to maintain this quality control a number of assays must be performed on the media before an IVF cycle begins. Assays are also important in evaluating and determining components that are detrimental for a defined medium to produce high developmental potential for IVF-derived embryos. With the required shelf life of embryo culture medium for human IVF laboratories being no longer than 1 to 2 weeks stored at 4°C (Purdy, 1982; Quinn et al., 1984; Gerrity et al., 1992; McLendon, 1992), a problem arises in performing more than one assay on a single batch of culture medium. Recently, commercial culture media manufactures have started to investigate the potential of frozen-thawed culture media. At this stage, there is still very little information available on the stability of culture media after the freezing-thawing process. Therefore, it is important to evaluate the ability of
blastocyst development in culture media that has been frozen and/or stored for a period of time longer than 1 to 2 weeks.
CHAPTER III
EXPERIMENT I

IN VITRO DEVELOPMENT OF BOVINE EMBRYOS USING FROZEN-THAWED COMMERCIAL CULTURE MEDIUM AT -20°C

Introduction

Being able to freeze and store embryo culture media would create the possibility of extending the standard (recommended) shelf life (Purdy, 1982; Quinn et al., 1984; Gerrity et al., 1992; McLendon, 1992) from 1 to 2 weeks at 4°C to an extended period. Human infertility clinics make fresh culture media approximately every 2 weeks or receive shipments of commercial culture medium that is produced on average every 2 weeks.

Over the past century researchers have gone from Albert Brachet’s 1912 blood plasma culture medium (Brachet, 1912, 1913) to Whitten’s (1957) and Brinster’s (1963) improved culture conditions that led to the in vitro development of 2-cell mouse embryos. Although improvements were made, Alexandre (2001) stated it was obvious to the early researchers that experiments would be hard to reproduce when using a culture medium such as blood plasma or blood serum, which contained complex unknown components. Research has continued over the years to develop more defined culture media to help reduce the variability among laboratories and commercial media.

A primary step in having a successful in vitro fertilization laboratory is having an efficient embryo culture system in place. There are many factors involved when setting up a successful in vitro fertilization (IVF) culture protocol. One important aspect is the evaluation of the culture media before being used. Many researchers suggest using an animal assay to evaluate results before using embryo culture media for human IVF. It usually takes 4 to 5 days to complete an assay leaving enough time for only one IVF cycle with that batch of medium. This restraint permits no time for multiple IVF cycles to be conducted to compare within a single batch of culture medium.

Many human IVF clinics use animal embryos (e.g., mice) to serve as a reference model for their human IVF embryos, since it is unethical and in most states illegal to perform research on human embryos. Once an efficient animal culture protocol is in
place the human culture system can be monitored using the animal embryo culture system. Data from both culture systems can be monitored to ensure embryo quality in the laboratory. The ideal design would be to compare results from the same batch of culture medium to reduce variation; however, the limited shelf life of the media creates a problem. One human IVF cycle takes from 3 to 5 days, depending on the embryo stage at transfer for individual clinics, leaving fresh culture media only available for use in two cycles.

In addition, comparison of IVF results among clinics is difficult and data comparing the same batch of medium among a number of different IVF cycles is usually not available. Over the years, it has been suggested by clinicians that the prescribed 2-week storage limitation is unnecessary (Naz et al., 1986; Bernart et al., 1990; De Silva, 1993; Bell et al., 1995). Clinical observations suggest that there is no decreased embryo development found with in vitro-produced embryos cultured in media stored for time periods greater than 2 weeks.

Limited research has been reported on the effects of freezing embryo culture media. Van den Berg (1959), Van den Berg and Rose (1959) and Bernart et al. (1990) are among the few researchers who studied the effects of frozen-thawed embryo culture media on embryos or the frozen-thawed viability effects of components in the media. Van den Berg (1959) demonstrated that pH of the medium could be affected during freezing by the presence of sodium and potassium chlorides in solutions of phosphates. Van den Berg and Rose (1959) showed how ice and salt precipitation also could affect the pH during freezing of solutions of monosodium and disodium potassium phosphates. Naz et al. (1986) and Bernart et al. (1990) have reported that Ham’s F-10 medium can be stored refrigerated at 4°C or frozen at -20°C without affecting 2-cell mouse embryo development in vitro.

The storage of culture media creates advantages that do not exist with reproducing stock media every 2 weeks. A number of batches can be made at the beginning of each IVF cycle, tested with the 1-cell mouse embryo bioassay and those batches selected that yield the best bioassay results. The best batches are then stored in a refrigerator at 4°C and subsequently used for human embryo cultures during that cycle. This would minimize variability among culture media and reduce technician time and expenses. More importantly, cold storage of tested media would enable gametes
and embryos from a group of patients to be exposed to the same batch of culture medium increasing laboratory efficiency. This would also increase the number of IVF cycles exposed to the same treatment for comparison. The benefit would be a better understanding of the processes of in vitro development without the variability between different batches of culture media.

The objective of this study was to test fresh and frozen-thawed human commercial culture medium at -20°C using IVF-derived bovine embryo development.

**Materials and Methods**

**Experimental Design**

For this experiment the experimental design originated from the commercial media company. This experiment was conducted to determine the developmental potential of IVF-derived bovine embryos cultured in a commercial medium frozen at -20°C and then frozen-stored for various time periods. IVF-derived bovine blastocysts cultured in frozen commercial medium were compared with IVF-derived bovine blastocysts cultured in the same fresh medium. Bovine 1-cell zygotes were shipped by Fedex Priority Overnight courier service to the Louisiana State University Agricultural Center Embryo Biotechnology Laboratory from a commercial source in a portable incubator. They were shipped in the afternoon arriving between 10:00 am and 1:00 pm the next day.

This experiment consisted of three treatment groups: Treatment A was a laboratory control CR1aa culture medium, Treatment B was a fresh commercial culture medium stored at 4°C and Treatment C was a frozen-thawed commercial culture medium frozen from periods of 3 days to 10 weeks before in vitro culture. Treatments A (n=6 replicates) and B (n=6 replicates) contained a total of 180 zygotes each. Treatment C (n=6 replicates, with 3 treatments per replicate) contained a total of 1,734 zygotes. For this experiment, Treatment B medium was recorded as Fresh 0 weeks. Treatment C was subdivided into six different frozen storage times; Frozen 3 days, Frozen 2 weeks, Frozen 4 weeks, Frozen 6 weeks, Frozen 8 weeks and Frozen 10 weeks. Frozen commercial medium had been previously frozen and stored at -20°C for its individual assigned time period by the commercial media supplier and shipped on dry ice to our laboratory. Over a period of 3 months 6 trials were performed, each one including a
group of IVF-derived embryos cultured in media from Treatment A, Treatment B and 3 groups cultured in Treatment C (Appendix D).

Zygotes in each replicate were randomly assigned to each of the three treatment groups and were cultured in the appropriate culture medium for 4 days. On day 4, CR1aa culture medium in Treatment A was supplemented with 5% fetal bovine serum (FBS) and the commercial culture medium was supplemented with 0.5 mM of glucose (G-6152, Sigma-Aldrich, Inc., St. Louis, MO). Culture was continued until day 8. Cleavage and blastocyst rates were assessed on day 4 and day 8 of in vitro culture, respectively, for all treatment groups.

**Experimental Procedure**

The fresh and frozen human commercial culture medium arrived on ice (wet and dry, respectively) by FedEx at the Louisiana State University Agricultural Center Embryo Biotechnology Laboratory (EBL) 1 day prior to its use. The name of this culture medium can not be disclosed for proprietary reasons, so for this thesis we have assigned a code name of GX2. Upon arrival, the fresh GX2 medium was stored overnight at 4°C and the frozen GX2 medium was thawed by placing it in a 37°C water bath then stored overnight at 4°C.

**Zygote Receipt and Preparation for Culture**

Bovine zygotes were received from a commercial supplier (BOMED, Inc., Madison, WI) in a portable incubator (Minitube®, Minitube America) at 39°C. Zygotes were shipped in groups of 50 per vial containing Tyrodes lactate HEPES medium (TL HEPES) and bovine serum albumin (BSA) (see Appendix B). Upon arrival vials were removed from the portable incubator and stored in a 5% CO₂ incubator in humidified air at 39°C until use. The zygotes were then removed from individual vials and placed into one Falcon® 35 x 10 mm plastic petri dish containing a TL HEPES medium (04-616F, Cambrex Bio Science Walkersville, Inc., Walkersville, MD). Two washes were made for each treatment medium (CR1aa, fresh and frozen-thawed GX2 commercial culture medium) creating a total of 10 Falcon® 35 x 10 mm plastic petri dishes. All the media had been previously incubated at 39°C to allow proper temperature and CO₂ equilibration. Zygotes were then washed twice in respective treatment medium and 15 zygotes were randomly selected and placed in each 75 µl culture droplet in a 35 x 10 mm
dish containing the corresponding medium. The number of 1-cell, 2-cell and 2- to 8-cell embryos were recorded before zygotes/embryos were placed into in vitro culture.

Media Preparation and Use

All chemicals were purchased from Sigma-Aldrich, Inc. (St. Louis, MO) unless indicated otherwise. The laboratory control culture medium (CR1aa) has been used for more than 2 years in our laboratory and was prepared as described previously by Rosenkrans and First (1994). The CR1aa stock solution was prepared by adding 114.7 mM NaCl (S-5886), 1.6 mM KCl (P-5405), 0.39 mM pyruvic acid (P-4562), 26.2 mM NaHCO₃ (S-8875), 2.5 mM L(+)-lactic acid (L-4388), 0.5195 mM glycine (G-8790), 0.5051 mM L-alanine (A-7469) and 0.2 ml of 0.5% phenol red per 100 ml of Milli-Q water. The CR1aa stock solutions were filtered with a 0.2 µm Acrodisc® sterile filter (PN 4612, Pall Corporation, East Hills, NY) and stored at 4°C for ≤3 months.

The control in vitro culture medium CR1aa for day 1 through day 4 was prepared weekly by combining 9.5 ml CR1aa stock solution, 2% (v/v) of Basal Medium Eagle (BME) amino acid solution (50X, B-6766), 1% (v/v) of Modified Eagle Medium (MEM) amino acid solution (10X, 11140-050, Gibco Laboratories, Grand Island, NY), 50 mg/ml of gentamycin (15750-060, Gibco Laboratories), 1.1 mM L-Glutamine (G-5763) and 3 mg/ml of BSA (A-7511). Medium was filtered with a 0.2 µm sterile filter (Acrodisc® Syringe Filters with Supor® Membrane). In Falcon® 35 x 10 mm plastic petri dishes, four 75 µl droplets were made and covered with warmed (39°C) medical grade mineral oil (330779) then placed in an incubator at 39°C for 45 to 60 minutes to allow medium to equilibrate.

On the day of use both fresh and frozen-thawed GX2 culture medium was aliquoted into 20 ml and supplemented with 0.3% of BSA (3 mg/ml, A-7511). No precipitate was observed in the frozen-thawed medium. The fresh and frozen-thawed GX2 medium was filtered with a 0.2 µm Acrodisc® sterile filter (PN 4612, Pall Corporation). Four 75 µl droplets were made with fresh and frozen-thawed medium in two Falcon® 35 x 10 mm plastic petri dishes for each treatment, covered with warmed (39°C) medical grade mineral oil and the dishes were then placed in an incubator at 39°C for 45 to 60 minutes to allow for proper temperature and CO₂ equilibration.

On day 4, the CR1aa control culture medium to be used for the next 4 days of culture was prepared the same as for the day 1 through day 4 culture period but with the
addition of 5% fetal bovine serum (FBS). Medium was filtered with a 0.2 µm Acrodisc® sterile filter (PN 4612, Pall Corporation) and placed into a Falcon® 35 x 10 mm plastic petri dish containing four 75 µl droplets that were covered with warmed (39°C) medical grade mineral oil. Dishes were placed in an incubator at 39°C for 45 to 60 minutes to allow proper temperature and CO₂ equilibration.

Fresh and frozen-thawed GX2 medium was supplemented with 0.5 mM of glucose for culture from day 4 to day 8 of the experiment (as called for by the commercial company protocol). Both fresh and the frozen GX2 medium was aliquoted into 20 ml and supplemented with 180 mg of glucose (G-6152), then filtered with a 0.2 µm Acrodisc® sterile filter (PN 4612, Pall Corporation). Four 75 µl droplets, covered with warmed (39°C) medical grade mineral oil, were made in a Falcon® 35 x 10 mm plastic petri dish(s) for fresh commercial culture medium and frozen-thawed commercial culture medium. The Falcon® 35 x 10 mm plastic petri dishes were then placed in an incubator at 39°C for 45 to 60 minutes to allow proper temperature and CO₂ equilibration.

Embryo Culture

Once zygotes were received and washed in appropriate culture medium, 15 zygotes were randomly placed in each appropriate 75 µl droplet contained in Falcon® 35 x 10 mm plastic petri dishes. Prior to this the zygotes were evaluated for 1-cell, 2-cell and 2- to 8-cell embryos and the data were recorded. The culture dishes were gassed in a humidified culture chamber for 4 minutes with 90% N₂, 5% O₂ and 5% CO₂. The humidified chambers containing all treatments were then placed in a 39°C incubator to be cultured until day 4 of the experiment.

On day 4, FBS supplemented CR1aa control culture medium was equilibrated in a 39°C incubator. Once the medium was equilibrated, embryos were removed from day 1 through day 4 culture droplets and placed into day 4 through day 8 culture droplets. Embryos were evaluated for morphology and development, and the number of 1-cell, 2- to 8-cell and 8- to 16-cell stages were recorded. Those at the 1-cell stage were not transferred to new culture medium droplets. The embryos (n=15) were then placed in each 75 µl droplet covered with warmed (39°C) medical grade mineral oil and gassed for 4 minutes with 90% N₂, 5% O₂ and 5% CO₂. Humidified chambers were then placed in a 39°C incubator and embryos were cultured until day 8 of the experiment.
Fresh and frozen-thawed GX2 medium supplemented with glucose was equilibrated in a 39°C incubator. Once medium was equilibrated embryos were transferred from day 1 through day 4 culture droplets to corresponding day 4 through day 8 culture droplets. Embryos were evaluated for morphology and development and data were recorded. Development was grouped by 1-cell, 2- to 8-cell and 8- to 16-cell stages and the remaining 1-cell zygotes were removed from culture. The embryos (n=15) were placed in each 75 µl culture droplet covered with warmed (39°C) medical grade mineral oil and gassed in a humidified culture chamber for 4 minutes with 90% N₂, 5% O₂ and 5% CO₂. Humidified chambers were then placed in a 39°C incubator to be cultured until day 8 of the study.

At ~192 hours post-insemination embryos were evaluated for development and morphological quality. A sample of the day-8 blastocysts (n=~15%) in each culture treatment group were randomly selected, stained in propidium iodide (for trophoblast cells) and Hoechst 33342 (for ICM cells) and fixed to determine the total cell numbers per embryo. Embryos were evaluated and graded using the RED scores system as described previously (Ryan et al., 1992). Development was grouped by early blastocysts, expanded blastocysts, hatching blastocysts and hatched blastocysts.

**Statistical Analysis**

Variances in embryo development and overall morphology were statistically analyzed using X² procedure of SAS (SAS Institute, Inc., Cary, NC). RED scores values were analyzed using the Proc Mixed procedure of SAS (SAS Institute, Inc.). Variances in inner cell mass cells (ICM), trophoblasts cells and total cell counts per embryo compared among treatments were analyzed using the ANOVA procedure of SAS (SAS Institute, Inc.). A P<0.05 value was considered statistically significant in this study. Descriptive statistics were analyzed using SigmaStat Statistical Software Version 2.0.

**Results**

**CR1aa Control Culture Medium**

Treatment A, the laboratory control culture medium, was replicated six times from April 30th to July 9th each time consisting of the same protocol. The percentages of 8- to 16-cell IVF-derived bovine embryos that developed in CR1aa medium at day 4 of culture
and blastocysts on day 8 of culture are shown in Figure 3.1a and Figure 3.1b, respectively. A mean of 43.0% of the embryos developed to the 8- to 16-cell stage on day 4 of in vitro culture, whereas, the blastocyst development on day 8 was 29.0%. There was no significant difference found among any of the six replicates for development of the 8- to 16-cell stage embryos and among the replicates at the blastocyst stage (Table 3.1). An example of day-8 CR1aa embryo development is shown in Figure 3.2.

Embryo development was statistically analyzed further by evaluating the variation of early blastocysts (EB), expanded blastocysts (XB), hatched blastocysts (Hatched) and hatching blastocysts (Hatching) of each replication (Figure 3.3). The percentages of day-8 IVF-derived bovine embryos that developed to the early blastocyst (EB), expanded blastocyst (XB) and hatching blastocyst (Hatching) stages in a control CR1aa culture medium are shown in Figure 3.4. The mean number of embryos that reached the early blastocyst stage of development was 41.0%, expanded blastocyst stage of development was 35.0%, hatching blastocyst stage of development was 22.0% and hatched stage of development was 2.0%.

When Replicate 1 was evaluated for further development, of the 30.0% total day-8 blastocysts, 33.3% of IVF-derived embryos cultured in control medium developed to early blastocysts, 33.3% developed to expanded blastocysts, 22.2% developed to hatching blastocysts and 11.0% developed to hatched blastocysts. When Replicate 2 was evaluated for further development, of the 43.3% total day-8 blastocysts, 61.5% of these IVF-derived embryos cultured in control medium developed to early blastocysts, 31.0% developed to expanded blastocysts, 8.0% developed to hatching blastocysts and 0% developed to hatched blastocysts. When Replicate 3 was evaluated for further development, of the 36.7% total day-8 blastocysts, 9.0% of IVF-derived embryos cultured in control medium developed to early blastocysts, 64.0% developed to expanded blastocysts, 27.3% developed to hatching blastocysts and 0% developed to hatched blastocysts.

When Replicate 4 was evaluated for further development, of the 20.0% total day-8 blastocysts, 50.0% of IVF-derived embryos cultured in control medium developed to early blastocysts, 16.7% developed to expanded blastocysts, 33.3% developed to
Figure 3.1a. Percentage of 8- to 16-cell IVF-derived bovine embryos that developed on day 4 of in vitro culture in CR1aa control medium beginning on April 30th and ending on July 9th.
Figure 3.1b. Percentage of IVF-derived bovine embryos that developed into blastocysts on day 8 of in vitro culture in CR1aa control medium beginning on April 30th and ending on July 9th.
Table 3.1. Percentage of IVF-derived bovine embryos that developed in weekly replicates of CR1aa culture medium.

<table>
<thead>
<tr>
<th>Replicates*</th>
<th>Day-4 8- to 16-Cells (%)</th>
<th>Day-8 Blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>60.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>50.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>40.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>30.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>36.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean =</td>
<td>43.3</td>
<td>28.9</td>
</tr>
</tbody>
</table>

*Each replicate of 30 zygotes for a total of 180 zygotes per 6 replicates.  
<sup>a,b</sup>Values within columns that have different superscripts are statistically different at P<0.05.
Figure 3.2. Day-8 IVF-derived bovine blastocysts cultured in CR1aa control culture medium (10X magnification).

Figure 3.3. Day-8 IVF-derived bovine expanded and hatching blastocysts cultured in CR1aa control culture medium (10X magnification).
Figure 3.4. Percentage of final total (n=52) day-8 IVF-derived bovine embryos that developed to the early blastocyst (EB), expanded blastocyst (XB) and hatching blastocyst (Hatching) stage in CR1aa control culture medium beginning on April 30th and ending on July 9th.
hatching blastocysts and 0% developed to hatched blastocysts. When Replicate 5 was evaluated for further development, out of the 10.0% total day-8 blastocysts, 33.3% of IVF-derived embryos cultured in control medium developed to early blastocysts, 33.3% developed to expanded blastocysts, 33.3% developed to hatching blastocysts and 0% developed to hatched blastocysts. When Replicate 6 was evaluated for further development, out of the 33.3% total day-8 blastocysts, 60.0% of IVF-derived embryos cultured in control medium developed to early blastocysts, 30.0% developed to expanded blastocysts, 10.0% developed to hatching blastocysts and 0% developed to hatched blastocysts. There was no significant difference found among any of the six replicates within the same developmental stage. However, the validity of the test performed on the hatched blastocyst group is questionable due to the low number of embryos that reached this developmental stage.

**GX2 Commercial Culture Medium**

The percent cleavage (day 4) of 2- to 8-cell IVF-derived bovine embryos that developed during in vitro culture in fresh or frozen-thawed commercial culture medium is shown in Figure 3.5. The 1-cell zygotes that did not develop were discarded at this point of the culture period. When evaluated on day 4 for cleavage, Treatment B (Fresh 0 weeks) had 11.2% 1-cell embryos and 37.2% 2- to 8-cell embryos. Storage time period for Frozen 3 days was evaluated on day 4 for cleavage and recorded 14.3% 1-cell embryos and 37.7% 2- to 8-cell embryos. When storage time period for Frozen 2 weeks was evaluated cleavage was recorded as 11.7% 1-cell embryos and 34.3% 2- to 8-cell embryos. When storage time period for Frozen 4 weeks was evaluated cleavage was recorded as 14.0% 1-cell embryos and 40.3% 2- to 8-cell embryos. Storage time period for Frozen 6 weeks was evaluated on day 4 and cleavage was recorded as 10.0% 1-cell embryos and 46.0% 2- to 8-cell embryos. When storage time period for Frozen 8 weeks was evaluated cleavage was recorded as 16.7% 1-cell embryos and 42.0% 2- to 8-cell embryos. Finally, storage time period for Frozen 10 weeks was evaluated on day 4 and cleavage was 19.0% 1-cell embryos and 40.7% 2- to 8-cell embryos. There was no statistical difference in 2- to 8-cell embryo cleavage when comparing Fresh 0 weeks with any of the different storage period times of frozen commercial culture medium (Frozen 3 day, Frozen 2 weeks, Frozen 4 weeks, Frozen 6 weeks, Frozen 8 weeks and Frozen 10 weeks).
Figure 3.5. Percentage of cleaved IVF-derived bovine embryos that developed on day 4 of in vitro culture in fresh or frozen-thawed commercial culture medium beginning on April 30th and ending on July 9th. Values with different superscripts within the same cleavage stage are statistically different at P<0.05.
Development of 8- to 16-Cell Embryos in Culture

The percentage of 8- to 16-cell IVF-derived bovine embryos that developed during in vitro culture in fresh or frozen-thawed commercial culture medium is shown in Figure 3.6 and Table 3.2. In the commercial culture medium development was assessed on day 4 at the 8- to 16-cell stage for both Treatment B and Treatment C. When Treatment B, which contained six replicates of one storage time period (Fresh 0 weeks), was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in fresh commercial culture medium was 51.7%.

Treatment C contained six different storage time periods with various frozen treatments repeated three times in each of the six replicates performed. When the first storage time period of Treatment C, Frozen 3 days, was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in frozen-thawed commercial culture medium was 47.8%. The next storage treatment time evaluated, Frozen 2 weeks, showed that the percentage of 8- to 16-cell IVF-derived embryos that developed in frozen-thawed commercial culture medium was 53.8%. When storage time period for Frozen 4 weeks was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in frozen-thawed commercial culture medium was 45.4%.

When storage time period for Frozen 6 weeks was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in frozen-thawed commercial culture medium was 44.1%. When storage time period for Frozen 8 weeks was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in frozen-thawed commercial culture medium was 41.2%. When the final storage time period, Frozen 10 weeks, was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in frozen-thawed commercial culture medium was 40.4%.

There was no significant difference found between Treatment B (Fresh 0 weeks) when compared with storage time periods for Frozen 3 days, Frozen 2 weeks, Frozen 4 weeks, and Frozen 6 weeks. However, when Treatment B (Fresh 0 weeks) was compared with Frozen 8 weeks and then again with Frozen 10 weeks there was a significant difference (P<0.05) in development found between the Fresh and Frozen treatments of both groups. There was also a significant difference (P<0.05) in development found between storage times for Frozen 2 weeks versus Frozen 6 weeks, Frozen 8 weeks and Frozen 10 weeks and Frozen 4 versus Frozen 10 weeks.
Figure 3.6. Percentage of 8- to 16-cell IVF-derived bovine embryos that developed on day 4 of in vitro culture in fresh or frozen-thawed commercial culture medium beginning on April 30th and ending on July 9th.
Table 3.2. Percentage (±SE) of 8- to 16-cell IVF-derived bovine embryos that developed in fresh or frozen-thawed GX2 commercial culture medium.

<table>
<thead>
<tr>
<th>Medium Storage Time</th>
<th>8- to 16-Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh 0 weeks</td>
<td>51.7 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 3 days</td>
<td>47.8 ± 9&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 2 weeks</td>
<td>53.8 ± 5&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 4 weeks</td>
<td>45.4 ± 9&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 6 weeks</td>
<td>44.1 ± 7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 8 weeks</td>
<td>41.2 ± 11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 10 weeks</td>
<td>40.4 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Values with different superscripts are statistically different at P<0.05.
Blastocyst Development in Culture

The percentage of IVF-derived bovine embryos that developed into blastocysts during in vitro culture in fresh or frozen-thawed commercial culture medium is shown in Figure 3.7 and Table 3.3. In the commercial culture medium, development was assessed on day 8 at the blastocyst stage for both Treatment B and Treatment C (Figure 3.8). When Treatment B, which contained six replicates of the control storage time period (Fresh 0 weeks), was evaluated, the percentage of IVF-derived blastocysts that developed in fresh commercial culture medium was 28.4%. Treatment C contained six different storage time periods with various frozen treatments repeated three times in each of the six replicates performed. When the first storage time period of Treatment C, Frozen 3 days, was evaluated the percentage of IVF-derived blastocysts that developed in frozen-thawed commercial culture medium was 31.1%. The next storage treatment time evaluated, Frozen 2 weeks, found the percentage of IVF-derived blastocysts that developed in frozen-thawed commercial culture medium was 29.5%.

When storage time period for Frozen 4 weeks was evaluated, the percentage of IVF-derived blastocysts that developed in frozen-thawed commercial culture medium was 27.3%. When storage time period for Frozen 6 weeks was evaluated, the percentage of IVF-derived blastocysts that developed in frozen-thawed commercial culture medium was 26.4%. When storage time period for Frozen 8 weeks was evaluated, the percentage of IVF-derived blastocysts that developed in frozen-thawed commercial culture medium was 23.3%. When the final storage time period, Frozen 10 weeks, was evaluated, the percentage of IVF-derived blastocysts that developed in frozen-thawed commercial culture medium was 24.8%. There was no significant difference found between Treatment B (Fresh 0 weeks) and all the different storage time periods of Treatment C (Frozen 3 days, Frozen 2 weeks, Frozen 4 weeks, Frozen 6 weeks, Frozen 8 weeks and Frozen 10 weeks).

Embryo development was statistically analyzed further by evaluating the variation of early blastocysts, expanded blastocysts, hatched blastocysts and hatching blastocysts of each treatment group. The percentage of day-8 IVF-derived bovine blastocysts that developed to the early blastocyst, expanded blastocyst and hatching blastocyst stage in
Figure 3.7. Percentage of IVF-derived bovine embryos that developed into blastocysts on day 8 of in vitro culture in fresh or frozen-thawed commercial culture medium beginning on April 30th and ending on July 9th.
Table 3.3. Percentage (±SE) of IVF-derived bovine blastocysts that developed in fresh or frozen-thawed GX2 commercial culture medium.

<table>
<thead>
<tr>
<th>Medium Storage Time</th>
<th>Blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh 0 weeks</td>
<td>28.4 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 3 days</td>
<td>31.1 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 2 weeks</td>
<td>29.5 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 4 weeks</td>
<td>27.3 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 6 weeks</td>
<td>26.4 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 8 weeks</td>
<td>23.3 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 10 weeks</td>
<td>24.8 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values with different superscripts are statistically different at P<0.05.
fresh or frozen-thawed commercial culture medium is shown in Figure 3.9. An example of further embryo development in GX2 medium is shown in Figure 3.10 and Figure 3.11. When Treatment B (Fresh 0 weeks) was evaluated for further development, of the 28.4% total blastocysts, 66.7% of IVF-derived embryos cultured in fresh commercial culture medium developed to early blastocysts, 33.3% developed to expanded blastocysts, 0% developed to hatching blastocysts and 0% developed to hatched blastocysts.

When the first storage time period of Treatment C, Frozen 3 days, was evaluated for further development, of the 31.1% total blastocysts, 75.0% of IVF-derived embryos cultured in frozen-thawed commercial culture medium developed to early blastocysts, 17.9% developed to expanded blastocysts, 4.0% developed to hatching blastocysts and 4.0% developed to hatched blastocysts. When storage time period for Frozen 2 weeks was evaluated for further development, of the 29.5% total blastocysts, 64.0% of IVF-derived embryos cultured in frozen-thawed commercial culture medium developed to early blastocysts, 33.0% developed to expanded blastocysts, 3.0% developed to hatching blastocysts and 0% developed to hatched blastocysts. When
Figure 3.9. Percentage of final total (n=518) day-8 IVF-derived bovine blastocysts that developed to the early blastocyst (EB) and expanded blastocyst (XB) stage in fresh or frozen-thawed commercial culture medium beginning on April 30th and ending on July 9th.
Figure 3.10. Day-8 IVF-derived bovine expanded blastocyst cultured in frozen-thawed GX2 commercial culture medium (20X magnification).

Figure 3.11. Day-8 IVF-derived bovine blastocysts cultured in frozen-thawed GX2 commercial culture medium (10X magnification).
storage time period for Frozen 4 weeks was evaluated for further development, of the 27.3% total blastocysts, 71.0% of IVF-derived embryos cultured in frozen-thawed commercial culture medium developed to early blastocysts, 28.0% developed to expanded blastocysts, 1.0% developed to hatching blastocysts and 0% developed to hatched blastocysts. When storage time period for Frozen 6 weeks was evaluated for further development, of the 26.4% total blastocysts, 67.0% of IVF-derived embryos cultured in frozen-thawed commercial culture medium developed to early blastocysts, 33.0% developed to expanded blastocysts, 0% developed to hatching blastocysts and 0% developed to hatched blastocysts.

When storage time period for Frozen 8 weeks was evaluated for further development, of the 23.3% total blastocysts, 75.0% of IVF-derived embryos cultured in frozen-thawed commercial culture medium developed to early blastocysts, 25.0% developed to expanded blastocysts, 0% developed to hatching blastocysts and 0% developed to hatched blastocysts. When the final storage time period, Frozen 10 weeks, was evaluated for further development, of the 24.8% total blastocysts, 69.0% of IVF-derived embryos cultured in frozen-thawed commercial culture medium developed to early blastocysts, 28.0% developed to expanded blastocysts, 2.0% developed to hatching blastocysts and 1.0% developed to hatched blastocysts. There was no significant difference found between Treatment B (Fresh 0 weeks) and any of the different storage times of Treatment C (Frozen 3 days, Frozen 2 weeks, Frozen 4 weeks, Frozen 6 weeks, Frozen 8 weeks and Frozen 10 weeks) when evaluating the same developmental stage.

The mean RED scores for IVF-derived bovine embryos recorded on day 8 following in vitro culture in fresh or frozen-thawed commercial culture is shown in Figure 3.12 and Table 3.4. In the commercial culture medium, morphology on day 8 was assessed as well as development for both Treatment B and Treatment C. A RED score was assigned to each embryo based on morphology and stage of development. When Treatment B, which contained six replicates of one storage time period (Fresh 0 weeks), was evaluated, the mean value assigned to the early blastocysts of IVF-derived embryos that developed in fresh commercial culture medium was 1.70% and the mean value assigned to expanded blastocysts was 2.85%. An example of further embryo development to day 9 is shown in Figure 3.13.
Figure 3.12. Mean RED scores for IVF-derived bovine embryos recorded on day 8 following in vitro culture in fresh or frozen-thawed commercial culture medium beginning on April 30th and ending on July 9th.
Table 3.4. Mean RED scores (±SEM) recorded on day 8 following bovine embryo culture in fresh or frozen-thawed GX2 commercial culture medium.

<table>
<thead>
<tr>
<th>Medium Storage Time</th>
<th>Early Blastocysts</th>
<th>Expended Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh 0 weeks</td>
<td>1.70 ±0.01a</td>
<td>2.85 ±0.06a</td>
</tr>
<tr>
<td>Frozen 3 days</td>
<td>1.72 ±0.06a</td>
<td>2.89 ±0.11a</td>
</tr>
<tr>
<td>Frozen 2 weeks</td>
<td>1.69 ±0.05a</td>
<td>2.89 ±0.04a</td>
</tr>
<tr>
<td>Frozen 4 weeks</td>
<td>1.76 ±0.07a</td>
<td>2.95 ±0.03a</td>
</tr>
<tr>
<td>Frozen 6 weeks</td>
<td>1.74 ±0.01a</td>
<td>2.86 ±0.02a</td>
</tr>
<tr>
<td>Frozen 8 weeks</td>
<td>1.52 ±0.12a</td>
<td>2.82 ±0.04a</td>
</tr>
<tr>
<td>Frozen 10 weeks</td>
<td>1.70 ±0.02a</td>
<td>2.88 ±0.04a</td>
</tr>
</tbody>
</table>

*a,b* Mean values within columns that have different superscripts are statistically different at P<0.05.

*Embryos were evaluated on day 8 of culture.
Treatment C contained six different storage time periods with various frozen treatments repeated three times in each of the six replicates performed. When the first storage time period of Treatment C, Frozen 3 days, was evaluated, the mean value assigned to the early blastocysts of IVF-derived embryos that developed in frozen-thawed commercial culture medium was 1.72% and mean the value assigned to expanded blastocysts was 2.89%. When storage time period for Frozen 2 weeks was evaluated, the mean value assigned to the early blastocysts of IVF-derived embryos that developed in frozen-thawed commercial culture medium was 1.69% and the mean value assigned to expanded blastocysts was 2.89%. When storage time period for Frozen 4 weeks was evaluated, the mean value assigned to the early blastocysts of IVF-derived embryos that developed in frozen-thawed commercial culture medium was 1.76% and the mean value assigned to expanded blastocysts was 2.95%.

When storage time period for Frozen 6 weeks was evaluated, the mean value assigned to the early blastocysts of IVF-derived embryos that developed in frozen-thawed commercial culture medium was 1.74% and the mean value assigned to expanded blastocysts was 2.86%. When storage time period for Frozen 8 weeks was
evaluated, the mean value assigned to the early blastocysts of IVF-derived embryos that
developed in frozen-thawed commercial culture medium was 1.52% and the mean value
assigned to expanded blastocysts was 2.82%. When the final storage time period,
Frozen 10 weeks, was evaluated, the mean value assigned to the early blastocysts of
IVF-derived embryos that developed in frozen-thawed commercial culture medium was
1.70% and the mean value assigned to expanded blastocysts was 2.88%. There was no
significant difference among early blastocysts or among expanded blastocysts analyzed
between Treatment B (Fresh 0 weeks) and any of the different frozen storage time
periods of Treatment C (Frozen 3 days, Frozen 2 weeks, Frozen 4 weeks, Frozen 6
weeks, Frozen 8 weeks and Frozen 10 weeks).

The percent mean inner cell mass (ICM) and percent mean trophoblast cell
counts from day-8 IVF-derived bovine embryos cultured in fresh or frozen-thawed
commercial culture medium is shown in Table 3.5. After morphology and development
were recorded a sample of the day-8 blastocysts (n=~15) in each culture treatment group
were randomly selected, stained in propidium iodide (for trophoblast cells) and Hoechst
33342 (for ICM cells) and fixed to determine the total cell numbers per embryo (Figure
3.14 and Figure 3.15). When evaluated, Treatment B (Fresh 0 weeks) recorded a final
count of 45.4 ICM cells and 85.1 trophoblast cells. Storage time period for Frozen 3
days resulted in a final count of 39.0 ICM cells and 79.3 trophoblast cells. When storage
time period for Frozen 2 weeks was evaluated, a final count of 45.3 ICM cells and 79.2
trophoblast cells were recorded.

Storage time period for Frozen 4 weeks resulted in a final count of 50.5 ICM cells
and 75.4 trophoblast cells. When evaluated, storage time period for Frozen 6 weeks
recorded a final count of 49.4 ICM cells and 86.2 trophoblast cells. When storage time
period for Frozen 8 weeks was evaluated, a final count of 49.1 ICM cells and 90.1
trophoblast cells were recorded. Lastly, storage time period for Frozen 10 weeks
resulted in a final count of 36.5 ICM cells and 90.6 trophoblast cells. There was no
significant difference found between Treatment B (Fresh 0 weeks) and any of the storage
time periods of Treatment C (Frozen 3 days, Frozen 2 weeks, Frozen 4 weeks, Frozen 6
weeks, Frozen 8 weeks and Frozen 10 weeks) for neither ICM cell count nor trophoblast
cell count.
Table 3.5. Percent mean inner cell mass (±SEM) and percent mean trophoblast cell counts (±SEM) from day-8 IVF-derived bovine embryos cultured in fresh or frozen-thawed GX2 commercial medium.

<table>
<thead>
<tr>
<th>Medium Storage Time</th>
<th>ICM</th>
<th>Trophoblasts</th>
<th>Total Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh 0 weeks</td>
<td>45.4 ± 5.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.1 ±11.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>130.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 3 days</td>
<td>39.0 ±19.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.3 ±14.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>118.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 2 weeks</td>
<td>45.3 ±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.2 ±8.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>124.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 4 weeks</td>
<td>50.5 ±13.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.4 ±11.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>125.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 6 weeks</td>
<td>49.4 ±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.2 ±3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>135.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 8 weeks</td>
<td>49.1 ±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.1 ±7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>139.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Frozen 10 weeks</td>
<td>36.5 ±9.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.6 ±9.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>127.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values within columns with different superscripts are statistically different at P<0.05.
ICM=Inner cell mass.
15% of embryos were selected from each treatment group.
Figure 3.14. Day-8 IVF-derived bovine blastocyst cultured in fresh GX2 commercial culture medium stained with propidium iodide (for trophoblast cells) and Hoechst 33342 (for ICM cells) (10X magnification).

Figure 3.15. Day-8 IVF-derived bovine blastocyst cultured in frozen-thawed GX2 commercial culture medium stained with propidium iodide (for trophoblast cells) and Hoechst 33342 (for ICM cells) (10X magnification).
**Discussion**

Storage of embryo culture media for up to 2 weeks is currently the maximum amount recommended by Purdy (1982) and Quinn et al. (1984) and part of the guidelines of the American Fertility Society (AFS) for ART programs (Gerrity et al., 1992). AFS and the College of American Pathologists (CAP) partnering to form an accreditation program ensuring high quality control standards, such as limited media storage time (McLendon, 1992). However, others propose that the media can be refrigerator stored or frozen-stored for longer periods of time for use in human embryo culture (Naz et al., 1986; Bernart et al., 1990; De Silva, 1993; Bell et al., 1995).

The use of frozen-thawed media would enable more efficient use of a single batch of medium and thus, more consistent data may be gained from the same batch of culture medium. Costs can be lowered per cycle because a number of different IVF cycles can be produced within the same batch of medium. To discard medium that supports in vitro fertilization and early embryo development merely to adhere to arbitrary guidelines regarding storage limitations is wasteful (Bell et al., 1995). Furthermore, inordinately frequent preparation of medium is inefficient use of personnel and materials (Bell et al., 1995).

In this experiment, a control bovine IVF culture medium (CR1aa) was used to monitor our in vitro development system, which previously had consistent results in the production of bovine embryos in our laboratory. In a study by Rosenkrans and First (1994), the developmental rates of morula and blastocyst stage embryos are similar or slightly decreased to our developmental rates when the CR1aa stock medium was refrigerator stored at 4°C up to 3 months. In our study replicates were performed simultaneously with the fresh and frozen-thawed IVF procedures to monitor the process and quality control. No significant difference was found in the development of 8- to 16-cell stage and blastocyst stage embryos among the six replicates performed with the control CR1aa medium. When development to the early blastocyst, expanded blastocyst and hatching blastocyst stage was evaluated further there was no difference in any of the three developmental stages found when compared among replicates. The number of embryos that reached the hatched blastocyst stage, however, was likely not sufficient for a valid statistical test.
In this experiment, cleavage was divided into two groups and recorded as the number of 2- to 8-cell embryos on day 4 and the number of 8- to 16-cells recorded on day 4. When evaluating cleavage of fresh versus frozen-thawed medium there was no developmental difference found among embryos at the 2- to 8-cell stage.

We did find, however, the freezing of a human IVF commercial culture medium at -20°C resulted in a significant decrease of bovine 8- to 16-cell development on day 4 when the medium was frozen for 8 weeks as well as frozen for 10 weeks when compared with fresh commercial culture medium. There was no significant decrease in development of the 8- to 16-cell stage when compared with the control Fresh 0 weeks when commercial culture medium was frozen for 3 days, 2 weeks, 4 weeks or 6 weeks. In storage period time for Frozen 2 weeks, 8- to 16-cell stage embryo development was actually slightly increased than that of the fresh control at 0 weeks. The 8- to 16-cell stage has been reported as the in vitro developmental block in bovine embryos (Thibault, 1966; Brackett et al., 1982; Camous et al., 1984; First and Parrish, 1987; Aoyagi et al., 1990; Keskintepe et al., 1995). This point in embryo cell division is known as the maternal to zygotic transition and may be very susceptible to components in culture media. Keskintepe et al. (1995) indicated one of the factors attributed to bovine embryonic development is the transfer of embryotrophic factors or metabolism from the culture media. Eyestone and First (1989), as well as Gandolfi and Moor (1987) agree that specific energy substrates may be necessary or detrimental for development of bovine embryos at this stage. Kim et al. (1993) reported that bovine oocytes matured and fertilized in a defined, protein free medium developed to the blastocyst stage with almost the same efficiency that has been obtained in co-culture, however it is essential to include amino acids, phosphate, pyruvate and lactate in the medium and glucose is necessary for later stages of development.

The energy requirements of bovine embryos during cleavage stages have not been fully defined (Rexroad, 1989; Rieger, 1992), in part, because of the difficulty imposed by the 8- to 16-cell in vitro developmental block (Thibault, 1966; Brackett et al., 1982; Camous et al., 1984; First and Parrish, 1987; Aoyagi et al., 1990; Keskintepe et al., 1995). The coincidence of this environmentally induced phenomenon with the maternal to zygotic transition is a point of considerable discussion (Bavister, 1988; Crosby et al., 1988; First and Barnes, 1989).
In the present experiment, 8- to 16-cell development was evaluated before the commercial culture medium was supplemented with glucose, which may account for the delayed or declined development due to the lack of this energy substrate in the medium. There are varying opinions on the addition of glucose to bovine embryo culture media. Rosenkrans et al. (1993) and Takahashi and First (1992) reported that glucose may not be the best choice of energy substrate because bovine embryo development decreased or was inhibited when glucose was added to the culture media. Takahashi and First (1992) indicated that glucose is not only unnecessary but at 5.56 mM it causes detrimental effects on bovine embryonic development to the morula stage. In contrast, Wirtu et al. (2004) reported beneficial results when glucose was supplemented through the entire culture period of bovine embryos. In agreement, Pantaleon et al. (2001) and Comizzoli et al. (2003) indicated that although the mechanism by which glucose exerts its effects during embryo development is not fully elucidated, its effects are evident as early as the fertilization stage in mice and bovine.

Therefore, in the present study, it is unknown if 8- to 16-cell development was significantly decreased in medium frozen (-20°C) for 8 to 10 weeks because of the detrimental effects of the post-thaw medium or because of the different requirements needed by bovine embryos not provided by the human commercial culture medium.

In this study, there were no significant differences in bovine blastocyst development among any of the different storage time periods of the fresh and frozen-thawed commercial culture medium previously stored at -20°C. These results are in agreement with those reported for the frozen storage of Ham’s F-10 medium used for murine and human in vitro fertilization by Bernart et al. (1990). Initially, they found there was no significant difference in development of mouse embryos to the expanded and hatched blastocyst stage cultured in Ham’s F-10 medium frozen at -20°C for up to 6 months. Following the animal results, the frozen-thawed medium was applied to human IVF and the results were the same, there was no loss of quality of the frozen-stored Ham’s F-10 medium within the 3 month test period.

When looking at storage time periods Frozen 3 days and Frozen 2 weeks (as with 8- to 16-cell development) there was a pattern for bovine blastocyst development on day 8 to be increased over that of the control (Fresh 0 weeks) but these developmental rates were not significant from the control fresh medium. Bernart et al. (1990) also suggested
that there was a pattern for higher fertilization and pregnancy rates of the human IVF embryos cultured in the frozen-stored Ham's F-10 medium when compared with the nonfrozen medium but this was not significant.

We evaluated development further to the early blastocyst and expanded blastocyst stages and found there was no difference in either developmental stage when compared among the treatment groups. The number of embryos that reached the hatching blastocyst stage and hatched blastocyst stage was likely not sufficient for a valid statistical test. Bernart et al. (1990) reported similar results in murine embryos cultured in frozen-thawed Ham's F-10 medium, there was no significant difference in the rates of expanded and hatched blastocysts between the subgroups cultured in fresh or frozen (-20°C) medium stored for up to 6 months.

When evaluating RED scores of early blastocysts in the present study, no significant differences were found in the grades of IVF-derived bovine embryos among fresh medium and any of the frozen-thawed storage period times. There was also no significant difference in RED scores value among expanded blastocysts when evaluating fresh and any of the frozen-thawed storage period times. In contrast to our results, Ryan et al. (1992) reported a significant amount of variation in RED scores when heat stressed embryos were cultured in different heat stress treatments for different time periods.

When the cell counts of day-8 IVF-derived bovine embryos were evaluated there was no significant difference found when cultured in either the fresh or the frozen-thawed commercial culture medium. However, again there was a pattern for total cell count to be equal to or increased in storage time periods for Frozen 6 weeks, Frozen 8 weeks and Frozen 10 weeks. Although there was an increased pattern there was no significant difference in development between these frozen storage time periods and the fresh commercial culture medium.

These results indicate that freezing human commercial culture medium at -20°C results in decreased 8- to 16-cell development when embryos are cultured in medium that has been frozen for 8 to 10 weeks. This is problematic because the norm for most human fertility clinics has been to transfer embryos on day 3 or day 4 known as the “cleaving embryo” stage. Edwards et al. (1980) and Steptoe et al. (1980) pioneered the human IVF procedure by transferring embryos at the cleavage stage into the synchronized uterus of the human female. Research is now being done to extend the
human embryo culture period out to day 5 or day 6 for transfer in an effort to select for embryo viability (Barnes et al., 1995; Gardner et al., 1998; Jones and Trounson, 1999; Cruz et al., 1999; Behr et al., 1999; Schoolcraft et al., 1999; Milki et al., 2000; Gardner et al., 2000; Alper et al., 2001). Alper et al. (2001) proposed that the culture of human embryos to the blastocyst stage of development has been advocated to increase the efficiency of IVF by improving embryo selection for increased implantation potential, reducing the number of embryos transferred and lowering the incidence of multiple gestations. Although there may be future advantages, a large amount of the data available to date is limited primarily to patients with good prognosis (Gardner et al., 1998; Schoolcraft et al., 1999).

To our knowledge this study was the first to evaluate bovine embryos in a frozen-thawed human commercial culture medium. Due to the commercial company protocol glucose was supplemented on day 4; however, there is much controversy over the addition of glucose when culturing bovine embryos in vitro. In a previous study, conducted at our university, Wirtu et al. (2004) demonstrated that bovine embryos cultured in modified KSOM (mKSOM-20aa, which includes 0.2 mM of glucose) with the addition of 20 amino acids achieved normal blastocyst development rates (42%). The commercial culture medium used in the present study was very similar in composition to modified KSOM (Lawitts and Biggers, 1991b) with the addition of amino acids; however, in the present study the concentration of glucose supplemented to the commercial culture medium was 0.5 mM. Wirtu (2004) reported low frequency of day-7 blastocysts indicating slow embryonic development in mKSOM-20aa but the frequency increased and by day 9 of culture development reached 42%. These data are similar to our results indicating decreased or delayed embryonic development at the 8- to 16-cell stage of embryos cultured in commercial medium frozen at -20°C for 8 to 10 weeks. The slow development indicates reduced developmental competence as reported for bovine and other species (Massip et al., 1995; Bavister, 2002). Further research must be performed on human commercial culture medium to determine if there is a delay around the 8- to 16-cell stage due to bovine embryo development in an alternate culture medium or if there is a composition change in the frozen-thawed culture medium.

Since this experiment showed no decreased development in blastocysts at day 8 among any of the treatments, we can assume that GX2 commercial culture medium can
be frozen at -20°C for up to 10 weeks and used without any detrimental effects to development of day-8 bovine blastocysts. However, human embryos are not usually cultured to day 8 before transfer, but with ongoing research of human blastocyst transfer (Barnes et al., 1995; Gardner et al., 1998; Jones and Trounson, 1999; Cruz et al., 1999; Behr et al., 1999; Schoolcraft et al., 1999; Milki et al., 2000; Gardner et al., 2000; Alper et al., 2001) this information may be important when considering cleavage stage transfer versus blastocyst stage transfer. Further research is needed to evaluate the rate of development in this frozen-thawed medium to determine if there is composition change of the culture medium.
CHAPTER IV
EXPERIMENT II

IN VITRO DEVELOPMENT OF BOVINE EMBRYOS USING FROZEN-THAWED COMMERCIAL CULTURE MEDIUM CRYOPRESERVED AT -80°C

Introduction

Over the years technology has advanced in treating infertile couples, with in vitro fertilization (IVF) and embryo transfer (ET) becoming two of the most important methodologies. De Silva (1993) indicated that one of the most critical issues when setting up an IVF laboratory is how gametes and embryos are going to be maintained in the laboratory prior to patient transfer. Thus, laboratories must have the best available embryo culture system in place to maintain quality control. At the present time, it is recommended that culture media for embryo culture should be stored for no longer than 1 to 2 weeks at 4°C (Purdy, 1982; Quinn et al., 1984; Gerrity et al., 1992; McLendon, 1992). Preparing new media every 2 weeks is time consuming and expensive resulting in decreased efficiency for the laboratory. Naz et al. (1986) showed that storing Ham’s F-10 medium for up to 425 days at 4°C did not affect 2-cell mouse embryo development to blastocysts in culture. However, Silverman et al. (1987) and Davidson et al. (1988a) indicated that the 2-cell mouse embryo may not be sensitive to subtle changes in the culture medium used for human embryos and, therefore, may not have detected any differences between the freshly prepared and the stored culture media. In another study, Bernart et al. (1990) reported that Ham’s F-10 medium could be frozen without affecting 2-cell mouse embryo development in vitro or affecting pregnancy rates in women undergoing IVF. In this study, it should be noted that the sodium bicarbonate solution and Ham’s F-10 medium were frozen separately and mixed prior to use, adjusting for osmolarity if needed.

De Silva (1993) stated that it would be ideal if a culture medium, once prepared, could be simply stored at 4°C and used for embryo cultures without compromising the development of the embryo. An experiment was designed where 1-cell mouse embryos were cultured in Ham’s F-10 medium freshly prepared (Fresh) or stored up to 6 months in a refrigerator at 4°C (Stored), to determine if storing the medium at 4°C affects embryo
development in vitro. The results showed no significant difference in murine embryo development to early, expanded, hatched and hatching stages, whether embryos were cultured in the fresh or the stored medium. De Silva (1993) proposed that these findings indicated that Ham’s F-10 medium can be stored in a refrigerator at 4°C for up to 6 months without compromising 1-cell mouse embryo development in vitro. The results of this study are in agreement with those of Naz et al. (1986) and the two previous studies reported by Bernart et al. (1990) showing that Ham’s F-10 medium can be stored refrigerated (4°C) or frozen (-20°C) without affecting 2-cell mouse embryo development in vitro.

Freezing IVF culture media creates advantages that are not available with the recommended storage time of 1 to 2 weeks. The prolonged storage time allows for multiple tests and cross tests to be performed on a single batch of medium. One problem that arises with limited storage time of culture media is the inability to perform all the assays needed. The average time to perform an assay on a batch of culture medium is usually 4 to 5 days leaving only 1 week of usable time left to use the culture medium. Another quality control method is to compare results among human infertility clinics, however, when one batch is being used for only 2 weeks little data are generated for comparisons. The ability to freeze culture media increases the number of IVF cycles that can be performed with the same medium producing more data that could be analyzed.

Bernart et al. (1990) described a method for frozen storage of Ham’s F-10 medium that is very simple, easy to use and provides ‘ready-to-use’ medium (within a few minutes) for IVF at any time. The results of freezing Ham’s F-10 medium at -20°C and then thawing showed that there is no loss of quality for at least several months in the mouse embryo model and possibly the clinical experience in human IVF. Bernart et al. (1990) pointed out this prolonged period of availability of one single medium preparation offers the possibility of being able to make extensive quality tests in an animal model for a new medium preparation before using it in human IVF. Bell et al. (1995) indicated, that there was no data reported in the literature suggesting a specific time point at which Ham’s F-10 medium, used worldwide in human IVF programs, was no longer supportive for human pre-implantation embryos. Their results suggested that Ham’s F-10 medium could be stored at 4°C for at least 3 months and still provide adequate support for fertilization and early in vitro development of the 4-cell stage human embryo.
The objective of this study was to test fresh and frozen-thawed commercial culture medium at -80°C by evaluating IVF-derived bovine embryo development and quality.

**Materials and Methods**

**Experimental Design**

The experimental design originated from the commercial media company. This experiment was conducted to determine the developmental potential of IVF-derived bovine embryos cultured in a commercial medium frozen and stored at -80°C for 1 month and then stored at 4°C for various time periods for use in embryo culture. IVF-derived bovine blastocysts cultured in frozen commercial medium were then compared with IVF-derived bovine blastocysts cultured in the same fresh commercial medium. For this experiment bovine 1-cell zygotes were shipped by FedEx Priority Overnight to the Louisiana State University Agricultural Center Embryo Biotechnology Laboratory from a commercial source in a portable incubator. The zygotes were shipped in the afternoon and arrived between 10 am and 12 pm the next day.

There were three treatment groups in this experiment, Treatment A was a laboratory control CR1aa culture medium, Treatment B was a fresh commercial culture medium that was stored at 4°C for various time periods and Treatment C was a frozen-thawed commercial culture medium. Treatment A (n=12 replicates) contained a total of 1,124 zygotes and was replicated 12 times from January 13th to May 5th each time consisting of the exact same protocol. Treatment B (n=12 replicates, one or two treatments per replicate) with a total of 1,500 zygotes and Treatment C (n=11 replicates) with a total of 1,102 zygotes. For this experiment Treatment B was divided into three different storage time periods; Fresh 0 weeks, Fresh 1 week and Fresh 2 weeks. Treatment C was also divided into three different storage time periods; Frozen 0 weeks, Frozen 1 week and Frozen 2 weeks. Frozen medium had been previously frozen and stored for 1 month at -80°C then shipped on dry ice to our laboratory within 1 day, 1 week, or 2 weeks of its use. Over a period of 4 months 12 trials were performed, each one including a group of IVF-derived embryos cultured in media from Treatment A and Treatment C and a group(s) of embryos cultured in Treatment B (Appendix E).
Zygotes in each replicate were randomly assigned to each of the three treatment groups and were cultured in appropriate culture media from day 1 through day 4. CR1aa culture medium in Treatment A was supplemented with 5% fetal bovine serum (FBS) on day 4 and cultured until day 8 of the experiment. On day 4 the commercial culture medium in Treatments B and C was supplemented with 0.5 mM of glucose (G-6152, Sigma-Aldrich, Inc., St. Louis, MO) and cultured from day 4 to day 8 of the experiment. Cleavage and blastocyst rates were assessed on day 4 and day 8 of in vitro culture, respectively, for all treatment groups.

Experimental Procedure

The name of the commercial culture medium used in this experiment can not be disclosed for proprietary reasons, so for this thesis we have assigned a code name of GX2. The fresh and frozen GX2 medium arrived on ice (wet and dry, respectively) from the commercial media company by FedEx at the Louisiana State University Embryo Biotechnology Laboratory (EBL) and was stored at 4°C according to its allotted time period. Frozen medium had been previously frozen and stored for 1 month at -80°C then shipped on dry ice to our laboratory within 1 day, 1 week or 2 weeks of its use. The fresh medium was shipped on wet ice to our laboratory within 1 day, 1 week or 2 weeks of its use. The day of use frozen medium was removed from 4°C storage, where it had been placed after arrival. When removed from storage if frozen medium was partially thawed to complete the thawing process it was placed into a 37°C water bath.

Zygote Receipt and Preparation for Culture

Bovine zygotes were received in a portable incubator (Minitube®, Minitube America) at 39°C from a commercial supplier (BOMED, Inc., Madison, WI) (Figure 4.1). Zygotes were shipped in groups of 50 per vial containing Tyrodes lactate HEPES (TL HEPES) and bovine serum albumin (BSA) (see Appendix B). Upon arrival at the EBL the shipping vials were removed from the portable incubator and stored in a 5% CO₂ incubator in humidified air at 39°C until use. Zygotes were then removed from each individual vial and placed into one Falcon® 35 x 10 mm plastic petri dish containing a TL HEPES medium (04-616F, Cambrex Bio Science Walkersville, Inc., Walkersville, MD). Six to eight Falcon® 35 x 10 mm plastic petri dishes were made containing two washes for each treatment medium (CR1aa medium, fresh and frozen-thawed GX2 commercial culture medium). All media had been previously equilibrated in a CO₂ incubator at 39°C.
Zygotes were then washed twice in respective treatment medium and 15 zygotes were randomly selected and placed in each 75 µl culture droplet in a 35 x 10 mm plastic dish. Prior to placement into in vitro culture the number of 1-cell, 2-cell and 2- to 8-cell embryos were recorded.

Media Preparation and Use

All chemicals were purchased from Sigma-Aldrich, Inc. (St. Louis, MO) unless indicated otherwise. The CR1aa laboratory control culture medium has been used for more than 2 years in our laboratory and was prepared as described previously by Rosenkrans and First (1994). CR1aa stock solution was prepared by adding 114.7 mM NaCl (S-5886), 1.6 mM KCl (P-5405), 0.39 mM pyruvic acid (P-4562), 26.2 mM NaHCO₃ (S-8875), 2.5 mM L(+)-lactic acid (L-4388), 0.5195 mM glycine (G-8790) 0.5051 mM L-alanine (A-7469), and 0.2 ml of 0.5% phenol red per 100 ml of Milli-Q water. The CR1aa stock solutions were filtered and stored at 4°C for ≤3 months.

The CR1aa control in vitro culture medium for day 1 through day 4 was prepared on the day of use by combining 9.5 ml CR1aa stock solution, 2% (v/v) of Basal Medium Eagle (BME) amino acid solution (50X, B-6766), 1% (v/v) of Modified Eagle Medium (MEM) amino acid solution (10X, 11140-050, Gibco Laboratories, Grand Island, NY), 50
mg/ml of gentamycin (15750-060, Gibco Laboratories), 1.1 mM L-Glutamine (G-5763) and 3 mg/ml of BSA (A-7511). Medium was filtered with a 0.2 µm Acrodisc® sterile filter (PN 4612, Pall Corporation, East Hills, NY). In Falcon® 35 x 10 mm plastic petri dishes, four 75 µl droplets were made, covered with warmed (39°C) medical grade mineral oil (330779, Sigma-Aldrich, Inc.) and allowed to equilibrate by placing in an incubator at 39°C for 45 to 60 minutes.

On day 1 of the culture period both fresh and frozen-thawed GX2 medium was aliquoted into 20 ml and supplemented with 0.3% of BSA (3 mg/ml, A-7511). No precipitate was observed in the frozen-thawed commercial culture medium. Fresh and frozen-thawed GX2 culture medium was filtered with a 0.2 µm Acrodisc® sterile filter (PN 4612, Pall Corporation). Four 75 µl droplets were made with fresh and frozen-thawed medium in two Falcon® 35 x 10 mm plastic petri dishes for each treatment and covered with warmed medical grade mineral oil (39°C). The dishes were then allowed time to reach the proper temperature and CO2 equilibration by being placed in an incubator at 39°C for 45 to 60 minutes.

On day 4, the control culture medium (CR1aa) to be used for the next 4 days was prepared the same as for the day 1 through day 4 culture period but with the addition of 5% FBS. CR1aa culture medium was filtered with a 0.2 µm Acrodisc® sterile filter (PN 4612, Pall Corporation) and placed into two Falcon® 35 x 10 mm plastic petri dishes each containing four 75 µl droplets that were covered with warmed (39°C) medical grade mineral oil. Dishes were then allowed time for proper temperature and CO2 equilibration by being placed in an incubator at 39°C for 45 to 60 minutes.

Also on day 4, the fresh and frozen-thawed GX2 medium to be used for the next 4 days of culture was supplemented with glucose (0.5 mM, as called for by commercial company protocol). Fresh and frozen-thawed GX2 medium was aliquoted into 20 ml each and supplemented with 180 mg of glucose, then filtered with a 0.2 µm Acrodisc® sterile filter (PN 4612, Pall Corporation). Four 75 µl droplets, covered with warmed (39°C) medical grade mineral oil, were made in two Falcon® 35 x 10 mm plastic petri dishes for each fresh and frozen-thawed commercial culture medium. The Falcon® 35 x 10 mm plastic petri dishes were then allowed time to reach the proper temperature and CO2 equilibration by being placed in an incubator at 39°C for 45 to 60 minutes.
An osmolarity test was run on randomly selected batches of fresh and frozen-thawed GX2 commercial culture medium, as well as the CR1aa control. The media tested was strictly used for this test and not re-used for in vitro culture. The osmolarity of CR1aa stock, without the addition of bovine serum albumin (BSA) or fetal calf serum (FCS) was 267 Osm. The average osmolarity of the fresh GX2 commercial culture medium, without the addition of BSA or glucose was 258 Osm. The average osmolarity of the frozen-thawed GX2 commercial culture medium, without the addition of BSA or glucose, was 259 Osm.

Embryo Culture

Prior to placing into culture, zygotes were evaluated for the number of 1-cell, 2-cell and 2- to 8-cell embryos and the observations were recorded. Once zygotes were washed in appropriate culture medium, 15 zygotes were randomly placed in each appropriate 75 µl droplet contained in Falcon® 35 x 10 mm plastic petri dishes. The culture dishes were gassed in a humidified cultured chamber with 90% N₂, 5% O₂ and 5% CO₂ for 4 minutes. The humidified chamber containing each treatment was then placed in an incubator at 39°C to be cultured until day 4 of the experiment.

On day 4 of the culture period, FBS supplemented CR1aa control culture medium was equilibrated in an incubator at 39°C. Day-4 embryos were evaluated for morphology and development, and the number of 1-cell, 2- to 8-cell and 8- to 16-cell stages were recorded. Cleaving embryos were removed from day 1 through day 4 culture droplets and placed into day 4 through day 8 culture droplets. However, those at the 1-cell stage were not transferred to new culture medium droplets. The embryos (n=15) were placed in each 75 µl droplet covered with warmed (39°C) medical grade mineral oil and gassed with 90% N₂, 5% O₂ and 5% CO₂ for 4 minutes. Humidified chambers containing each treatment group were then placed in a 39°C incubator and embryos were cultured until day 8 of the experiment.

Also on day 4 of the culture period, glucose supplemented fresh and frozen-thawed GX2 medium was equilibrated in an incubator at 39°C. Day-4 embryos were evaluated for morphology and development and data were recorded. Development was grouped by 1-cell, 2- to 8-cell and 8- to 16-cell stages and the remaining 1-cell zygotes were removed from culture. Cleaving embryos were transferred from day 1 through day 4 culture droplets to corresponding day 4 through day 8 culture droplets. The embryos
(n=15) were placed in each 75 µl culture droplet covered with warmed (39°C) medical grade mineral oil and gassed in a humidified culture chamber with 90% N₂, 5% O₂ and 5% CO₂ for 4 minutes. Humidified chambers containing each treatment group were then placed in a 39°C incubator to be cultured until day 8 of the experiment.

At ~192 hours post-insemination embryos were evaluated for development and morphological quality. Embryos were evaluated and graded using the RED scores system as described previously (Ryan et al., 1992). Development was grouped by early blastocysts, expanded blastocysts, hatching blastocysts and hatched blastocysts.

**Statistical Analysis**

Variances in embryo development and overall morphology were statistically analyzed using X² procedure of SAS (SAS Institute, Inc., Cary, NC). RED scores were statistically analyzed using the proc mixed procedure of SAS (SAS Institute, Inc.). A P<0.05 value was considered statistically significant in this study. Descriptive statistics were analyzed using SigmaStat Statistical Software Version 2.0.

**Results**

**CR1aa Control Culture Medium**

The treatment A, laboratory control culture medium, was replicated 12 times from January 13ᵗʰ to May 5ᵗʰ consisting of the exact same protocol in each replicate. The percentage of 8- to 16-cell IVF-derived bovine embryos at day 4 of culture is shown in Figure 4.2a. Following is the percentage of IVF-derived bovine embryos that developed into blastocysts on day 8 of in vitro culture in CR1aa control medium (Figure 4.2b). A mean of 54.0% of the embryos in Treatment A developed to the 8- to 16-cell stage on day 4 of in vitro culture, whereas, the mean blastocyst development on day 8 was 27.0%.

When evaluating 8- to 16-cell stage embryo development on day 4 of in vitro culture there was a significant difference (P<0.05) found when comparing Replicate 1 (44.0%) with replicates 2 (16.4%), 6 (68.1%), 7 (62.6%), 9 (59.9%) and 12 (64.0%). When evaluating blastocyst stage development on day 8 of in vitro culture there was no significant difference found among any of the 12 replicates (Table 4.1). An example of embryo development in CR1aa control medium is shown in Figure 4.3.

Embryo development was statistically analyzed further by evaluating the variation of early blastocysts (EB), expanded blastocysts (XB), hatched blastocysts (Hatched) and
Figure 4.2a. Percentage of 8- to 16-cell IVF-derived bovine embryos that developed on day 4 of in vitro culture in CR1aa control medium beginning on January 13th and ending on May 5th.
Figure 4.2b. Percentage of IVF-derived bovine embryos that developed into blastocysts on day 8 of in vitro culture in CR1aa control medium beginning on January 13th and ending on May 5th.
Table 4.1. Percent IVF-derived bovine embryos that developed in weekly replicates of CR1aa culture medium.

<table>
<thead>
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<th>Replicates*</th>
<th>Embryo Stages</th>
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<tr>
<td></td>
<td>Day-4 8- to 16-Cells (%)</td>
<td>Day-8 Blastocysts (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>44.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>16.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>53.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>37.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>55.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>34.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>38.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>15.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>68.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>7</td>
<td>62.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>8</td>
<td>56.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>68.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>17.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>59.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>26.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>11</td>
<td>48.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>18.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>64.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mean =</td>
<td>52.9</td>
<td>26.9</td>
<td></td>
</tr>
</tbody>
</table>

*Total number of 1-cell embryos in the six replicates=1,125.
<sup>a,b</sup>Values within columns that have different superscripts are statistically different at P<0.05.
Figure 4.3. Day-8 bovine blastocysts and hatched blastocyst in CR1aa control culture medium (10X magnification).

hatching blastocysts (Hatching) of each replication. The percentages of day-8 IVF-derived bovine embryos that developed to the early blastocyst (EB), expanded blastocyst (XB) and hatching blastocyst (Hatching) stage in a control CR1aa culture medium are shown in Figure 4.4. The mean number of embryos that reached the early blastocyst stage of development was 55.0%, expanded blastocyst stage of development was 39.0%, hatching blastocyst stage of development was 2.0% and hatched blastocyst stage of development was 4.0%.

When Replicate 1 was evaluated for further development, of the 26.0% total day-8 blastocysts, 46.2% of the IVF-derived embryos cultured in control medium developed to early blastocysts, 46.2% developed to expanded blastocysts, 0% developed to hatching blastocysts and 8.0% developed to hatched blastocysts. When Replicate 2 was evaluated for further development, of the 24.0% total day-8 blastocysts, 69.0% of the IVF-derived embryos cultured in control medium developed to early blastocysts, 31.0% developed to expanded blastocysts, 0% developed to hatching blastocysts and 0% developed to hatched blastocysts. After evaluating Replicate 3 for further development, of the 37.5% total day-8 blastocysts, 53.0% of the IVF-derived embryos cultured in
Figure 4.4. Percentage of final total (n=309) day-8 IVF-derived bovine embryos that developed to the early blastocyst (EB), expanded blastocyst (XB) and hatching blastocyst (Hatching) stage in CR1aa control culture medium beginning on January 13th and ending on May 5th.
control medium had developed to early blastocysts, 39.0% had developed to expanded blastocysts, 3.0% had developed to hatching blastocysts and 5.0% had developed to hatched blastocysts. When Replicate 4 was evaluated for further development, of the 35.0% total day-8 blastocysts, 27.0% of the IVF-derived embryos cultured in control medium developed to early blastocysts, 59.0% developed to expanded blastocysts, 0% developed to hatching blastocysts and 14.0% developed to hatched blastocysts. After evaluating Replicate 5 for further development, of the 16.0% total day-8 blastocysts, 38.0% of the IVF-derived embryos cultured in control medium had developed to early blastocysts, 46.0% had developed to expanded blastocysts, 8.0% had developed to hatching blastocysts and 8.0% had developed to hatched blastocysts.

When Replicate 6 was evaluated for further development, of the 36.0% total day-8 blastocysts, 73.0% of the IVF-derived embryos cultured in control medium developed to early blastocysts, 24.0% developed to expanded blastocysts, 3.0% developed to hatching blastocysts and 0% developed to hatched blastocysts. When Replicate 7 was evaluated for further development, of the 24.0% total day-8 blastocysts, 64.0% of the IVF-derived embryos cultured in control medium developed to early blastocysts, 33.0% developed to expanded blastocysts, 3.0% developed to hatching blastocysts and 0% developed to hatched blastocysts. After evaluating Replicate 8 for further development, of the 39.0% total day-8 blastocysts, 62.2% of the IVF-derived embryos cultured in control medium had developed to early blastocysts, 31.1% had developed to expanded blastocysts, 2.2% developed to hatching blastocysts and 4.4% had developed to hatched blastocysts.

When Replicate 9 was evaluated for further development, of the 18.0% total day-8 blastocysts, 69.0% of the IVF-derived embryos cultured in control medium developed to early blastocysts, 31.0% developed to expanded blastocysts, 0% developed to hatching blastocysts and 0% developed to hatched blastocysts. After Replicate 10 was evaluated for further development, of the 27.0% total day-8 blastocysts, 65.0% of the IVF-derived embryos cultured in control medium had developed to early blastocysts, 35.0% had developed to expanded blastocysts, 0% had developed to hatching blastocysts and 0% had developed to hatched blastocysts. After evaluating Replicate 11 for further development, of the 19.0% total day-8 blastocysts, 37.0% of the IVF-derived embryos cultured in control medium had developed to early blastocysts, 53.0% had developed to
expanded blastocysts, 5.0% had developed to hatching blastocysts and 5.0% had developed to hatched blastocysts. When Replicate 12 was evaluated for further development, of the 23.0% total day-8 blastocysts, 57.0% of the IVF-derived embryos cultured in control medium developed to early blastocysts, 35.0% developed to expanded blastocysts, 4.0% developed to hatching blastocysts and 4.0% developed to hatched blastocysts.

When analyzed, there was a significant difference in early blastocysts development detected when Replicate 1 was compared with replicates 2, 4, 6, 7, 9 and 10. There was no significant difference in expanded blastocysts development among any of the 12 replicates. There was also no significant difference in hatching blastocysts development and hatched blastocysts (development) among replicates, however the validity of the test is questionable due to the low number of embryos that reached these developmental stages. An example of hatching and hatched embryo development in CR1aa control medium is shown in Figure 4.5.

GX2 Commercial Culture Medium

The percent cleavage (day 4) of 2- to 8-cell IVF-derived bovine embryos that developed during in vitro culture in fresh or frozen-thawed commercial culture medium is shown in Figure 4.6. The 1-cell zygotes that did not develop were discarded at this point of the culture period. On day 4 each of the three different storage time periods (Fresh 0 weeks, Fresh 1 week and Fresh 2 weeks) were evaluated for cleavage. When storage time period for Fresh 0 weeks was evaluated on day 4, cleavage was recorded as 15.7% 1-cell embryos and 23.0% 2- to 8-cell embryos. When storage time period for Fresh 1 week was evaluated on day 4, cleavage was recorded as 10.0% 1-cell embryos and 28.0% 2- to 8-cell embryos. When storage time period for Fresh 2 weeks was evaluated on day 4, cleavage was recorded as 13.5% 1-cell embryos and 24.0% 2- to 8-cell embryos. When storage time period for Frozen 0 weeks was evaluated on day 4, cleavage was recorded as 13.0% 1-cell embryos and 26.0% 2- to 8-cell embryos. When storage time period for Frozen 1 week was evaluated on day 4, cleavage was recorded as 10.0% 1-cell embryos and 39.0% 2- to 8-cell embryos. When storage time period for Frozen 2 weeks was evaluated on day 4, cleavage was recorded as 18.0% 1-cell embryos and 19.5% 2- to 8-cell embryos.
Figure 4.5. Bovine embryos in CR1aa control culture medium. A. Day-8 hatched blastocyst at 20X magnification. B. A day-9 hatching blastocyst (arrow) at 20X magnification.
Figure 4.6. Percentage of cleaved IVF-derived bovine embryos that developed on day 4 of in vitro culture in fresh or frozen-thawed commercial culture medium beginning on January 13th and ending on May 5th. a,b Values with different superscripts within the same cleavage stage are significantly different at P<0.05.
When evaluating cleavage rates there was a significant difference (P<0.05) in 2- to 8-cell development when comparing embryos cultured in fresh control commercial culture medium (0 weeks) with Fresh 1 week and Frozen 1 week. There was also a significant difference in cleavage found when comparing stored and frozen treatments among themselves. A significant difference (P<0.05) was found between storage time periods for Fresh 1 week compared with Fresh 2 weeks, Frozen 0 weeks and Frozen 2 weeks, as well as, Fresh 2 weeks versus Frozen 1 week, Frozen 0 weeks versus Frozen 1 week and Frozen 1 week versus Frozen 2 weeks.

Development of 8- to 16-Cell Embryos in Culture

The percentages of 8- to 16-cell IVF-derived bovine embryos that developed on day 4 during in vitro culture in fresh or frozen-thawed commercial culture medium are shown in Figure 4.7 and Table 4.2. In the commercial culture medium development was assessed on day 4 at the 8- to 16-cell stage for both Treatment B and Treatment C (Figure 4.8). Treatment B contained three different storage time periods (Fresh 0 weeks, Fresh 1 week and Fresh 2 weeks, one or two treatments per replicate) used among the 12 replicates completed. When the first storage time period of Treatment B, Fresh 0 weeks, was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in fresh commercial culture medium was 57.0%. When storage time period for Fresh 1 week was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in fresh commercial culture medium was 52.7%. When the third storage time period, Fresh 2 weeks, was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in fresh commercial culture medium was 61.3%.

Treatment C contained three different storage time periods (Frozen 0 weeks, Frozen 1 week and Frozen 2 weeks) used among the 11 replicates completed. When the first storage time period of Treatment C, Frozen 0 weeks, was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in frozen-thawed commercial culture medium was 58.2%. The next storage treatment time evaluated, Frozen 1 week, showed the percentage of 8- to 16-cell IVF-derived embryos that developed in frozen-thawed commercial culture medium was 47.7%. When storage time period for Frozen 2 weeks was evaluated, the percentage of 8- to 16-cell IVF-derived embryos that developed in frozen-thawed commercial culture medium was 65.3%.
Figure 4.7. Percentage of 8- to 16-cell IVF-derived bovine embryos that developed on day 4 of in vitro culture in fresh or frozen-thawed commercial culture medium beginning on January 13th and ending on May 5th.
Table 4.2. Percentage (±SE) of 8- to 16-cell bovine embryo development in fresh or frozen-thawed GX2 commercial culture medium.

<table>
<thead>
<tr>
<th>Medium Storage Time</th>
<th>8- to 16-Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh 0 weeks</td>
<td>57.0 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fresh 1 week</td>
<td>52.7 ± 10&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fresh 2 weeks</td>
<td>61.3 ± 4&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 0 weeks</td>
<td>58.2 ± 3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 1 week</td>
<td>57.7 ± 11&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 2 weeks</td>
<td>65.3 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values with different superscripts are significantly different at P<0.05.
There was a significant outlier detected in storage treatment Frozen 1 week. Due to the fact that this one (outlier) group of 8- to 16-cell embryos were the only group with a substantial decrease in development a decision was made to remove their developmental rates from the mean of the Frozen 1 week storage time period. When this was done there was no significant difference found between Fresh 0 weeks when compared with Fresh 1 week, Fresh 2 weeks, Frozen 0 weeks and Frozen 1 week. However, there was a significant difference (P<0.05) found when Fresh 0 weeks was compared with the storage time period for Frozen 2 week. There was also a significant difference (P<0.05) detected when comparing the frozen-thawed storage time periods among themselves. The storage time periods that were found to be significantly different included Fresh 1 week versus Fresh 2 weeks and Frozen 2 weeks, Fresh 2 weeks versus Frozen 1 week, Frozen 0 weeks versus Frozen 1 week and Frozen 1 week versus Frozen 2 weeks.

Blastocyst Development in Culture

The percentage of IVF-derived bovine embryos that developed into blastocysts on day 8 during in vitro culture in fresh or frozen-thawed commercial culture medium is
shown in Figure 4.9 and Table 4.3. In the commercial culture medium, development was assessed on day 8 at the blastocyst stage of development for both Treatment B and Treatment C (Figure 4.10). Treatment B contained three different storage time periods (Fresh 0 weeks, Fresh 1 week and Fresh 2 weeks, one or two treatments per replicate) used among the 12 replicates completed. When the first storage time period of Treatment B, Fresh 0 weeks, was evaluated, the percentage of IVF-derived blastocysts that developed in fresh commercial culture medium was 14.2%. When storage time period for Fresh 1 week was evaluated, the percentage of IVF-derived blastocysts that developed in fresh commercial culture medium was 18.5%. When the third storage time period, Fresh 2 weeks, was evaluated, the percentage of IVF-derived blastocysts that developed in fresh commercial culture medium was 15.4%.

Treatment C contained three different storage time periods (Frozen 0 weeks, Frozen 1 week and Frozen 2 weeks) used among the 11 replicates completed. When the first storage time period of Treatment C, Frozen 0 weeks, was evaluated, the percentage of IVF-derived blastocysts that developed in frozen-thawed commercial culture medium was 13.9%. The next storage treatment time evaluated, Frozen 1 week, showed the percentage of IVF-derived blastocysts that developed in frozen-thawed commercial culture medium was 10.0%. When storage time period for Frozen 2 weeks was evaluated, the percentage of IVF-derived blastocysts that developed in frozen-thawed commercial culture medium was 17.7%.

There was no significant difference found when Fresh 0 weeks was compared with any of the other storage time periods. There was a significant difference (P<0.05) in blastocyst development found when comparing Fresh 1 week versus Frozen 1 week, Fresh 2 weeks versus Frozen 1 week and Frozen 1 week versus Frozen 2 weeks.

Embryo development was statistically analyzed further by evaluating the variation of early blastocysts, expanded blastocysts, hatched blastocysts and hatching blastocysts of each treatment group (4.11). The percentages of day-8 IVF-derived bovine blastocysts that developed to the early blastocyst and expanded blastocyst stage in fresh or frozen-thawed commercial culture medium are shown in Figure 4.12. When Fresh 0 weeks was evaluated for further development, of the 14.2% total blastocysts, 72.0% of IVF-derived embryos cultured in fresh commercial culture medium developed to early blastocysts, 28.0% developed to expanded blastocysts, 0% developed to hatching
Figure 4.9. Percentage of IVF-derived bovine embryos that developed into blastocysts on day 8 of in vitro culture in fresh or frozen-thawed commercial culture medium beginning on January 13th and ending on May 5th.
Table 4.3. Percentage (±SE) of IVF-derived bovine blastocysts that developed in fresh or frozen-thawed GX2 commercial culture medium.

<table>
<thead>
<tr>
<th>Medium Storage Time</th>
<th>Blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh 0 weeks</td>
<td>14.2 ± 3\textsuperscript{a}</td>
</tr>
<tr>
<td>Fresh 1 week</td>
<td>18.5 ± 4\textsuperscript{a}</td>
</tr>
<tr>
<td>Fresh 2 weeks</td>
<td>15.4 ± 3\textsuperscript{a}</td>
</tr>
<tr>
<td>Frozen 0 weeks</td>
<td>13.9 ± 2\textsuperscript{a}</td>
</tr>
<tr>
<td>Frozen 1 week</td>
<td>10.0 ± 4\textsuperscript{a}</td>
</tr>
<tr>
<td>Frozen 2 weeks</td>
<td>17.7 ± 2\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b}Values with different superscripts are significantly different at P<0.05.
Figure 4.10. Day-8 expanded blastocyst in fresh GX2 commercial culture medium (20X magnification).

Figure 4.11. Day-8 hatching blastocyst in fresh GX2 commercial culture medium (20X magnification).
Figure 4.12. Percentage of final total (n=388) day-8 IVF-derived bovine blastocysts that developed to the early blastocyst (EB) and expanded blastocyst (XB) stage in fresh or frozen-thawed commercial culture medium beginning on January 13th and ending on May 5th.
blastocysts and 0% developed to hatched blastocysts. When Fresh 1 week was evaluated for further development, of the 18.5% total blastocysts, 62.0% of IVF-derived embryos cultured in fresh commercial culture medium developed to early blastocysts, 37.0% developed to expanded blastocysts, 1.0% developed to hatching blastocysts and 0% developed to hatched blastocysts. When Fresh 2 weeks was evaluated for further development, of the 15.4% total blastocysts, 74.0% of IVF-derived embryos cultured in fresh commercial culture medium developed to early blastocysts, 23.0% developed to expanded blastocysts, 3.0% developed to hatching blastocysts and 0% developed to hatched blastocysts.

When Frozen 0 weeks was evaluated for further development, of the 13.9% total blastocysts, 70.0% of IVF-derived embryos cultured in frozen-thawed commercial culture medium developed to early blastocysts, 26.0% developed to expanded blastocysts, 2.0% developed to hatching blastocysts and 2.0% developed to hatched blastocysts. When Frozen 1 week was evaluated for further development, of the 10.0% total blastocysts, 62.0% of IVF-derived embryos cultured in frozen-thawed commercial culture medium developed to early blastocysts, 36.0% developed to expanded blastocysts, 2.0% developed to hatching blastocysts and 0% developed to hatched blastocysts. When Frozen 2 weeks was evaluated for further development, of the 17.7% total blastocysts, 64.0% of IVF-derived embryos cultured in frozen-thawed commercial culture medium developed to early blastocysts, 32.0% developed to expanded blastocysts, 4.0% developed to hatching blastocysts and 0% developed to hatched blastocysts. No significant difference was detected when comparing early blastocyst or expanded blastocyst development between storage time period Fresh 0 weeks and the remaining storage time periods (Fresh 1 week, Fresh 2 weeks, Frozen 0 weeks, Frozen 1 week and Frozen 2 weeks).

The mean RED scores for IVF-derived bovine embryos recorded on day 8 following in vitro culture in fresh or frozen-thawed commercial culture medium is shown in Figure 4.13 and Table 4.4. In the commercial culture medium, morphology on day 8 was assessed as well as development for all storage time periods in both the fresh and frozen-thawed culture medium. A RED score was assigned to each embryo based on morphology and stage of development (Figure 4.14). When storage time period for
Figure 4.13. Mean RED scores for IVF-derived bovine embryos recorded on day 8 following in vitro culture in fresh or frozen-thawed commercial culture medium beginning on January 13\textsuperscript{th} and ending on May 5\textsuperscript{th}. 
Table 4.4. Mean RED scores (±SEM) recorded on day 8 following bovine embryo culture in fresh or frozen-thawed GX2 commercial culture medium.

<table>
<thead>
<tr>
<th>Medium Storage Time</th>
<th>Embryos*</th>
<th>Early Blastocysts</th>
<th>Expanded Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh 0 weeks</td>
<td></td>
<td>1.86 ±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.98 ±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fresh 1 week</td>
<td></td>
<td>1.86 ±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.91 ±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fresh 2 weeks</td>
<td></td>
<td>1.81 ±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.94 ±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 0 weeks</td>
<td></td>
<td>1.86 ±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.96 ±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 1 week</td>
<td></td>
<td>1.88 ±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.93 ±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen 2 weeks</td>
<td></td>
<td>1.84 ±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.93 ±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Mean values within columns that have different superscripts are significantly different at P<0.05.

*Embryos were evaluated on day 8 of culture.
Figure 4.14. Day-8 bovine embryos in frozen-thawed GX2 commercial culture medium. 
A. Day-8 blastocysts at 20X magnification. B. Day-8 blastocysts and expanded blastocyst at 20X magnification.
Fresh 0 weeks was evaluated, the mean RED score for the early blastocysts of IVF-derived embryos that developed in fresh commercial culture medium was 1.86% and the mean RED score for the expanded blastocysts was 2.98%. When the next storage time period, Fresh 1 week, was evaluated, the mean RED score for the early blastocysts of IVF-derived bovine embryos that developed in fresh commercial culture medium was 1.86% and the mean RED score for the expanded blastocysts was 2.91%. When storage time period for Fresh 2 weeks was evaluated, the mean RED score for the early blastocysts of IVF-derived embryos that developed in fresh commercial culture medium was 1.81% and the mean RED score for the expanded blastocysts was 2.94%.

When storage time period for Frozen 0 weeks was evaluated, the mean RED score for the early blastocysts of IVF-derived embryos that developed in frozen-thawed commercial culture medium was 1.86% and the mean RED score for the expanded blastocysts was 2.96%. When storage time period for Frozen 1 week was evaluated, the mean RED score for the early blastocysts of IVF-derived embryos that developed in frozen-thawed commercial culture medium was 1.88% and the mean RED score for the expanded blastocysts was 2.93%. When storage time period for Frozen 2 weeks was evaluated, the mean RED score for the early blastocysts of IVF-derived embryos that developed in frozen-thawed commercial culture medium was 1.84% and the mean RED score for the expanded blastocysts was 2.93%. There was no significant difference among early blastocysts or among expanded blastocysts analyzed between the control storage time period Fresh 0 weeks and any of the other fresh or frozen-thawed storage time periods (Fresh 1 week, Fresh 2 weeks, Frozen 0 weeks, Frozen 1 week and Frozen 2 weeks).

**Discussion**

As in vitro fertilization and embryo transfer (IVF/ET) becomes more accepted by infertile patients, one of the most critical aspects to consider is how the gametes and embryos are to be maintained in the laboratory until they are transferred to the patient. Therefore, it very important that the best culture media available is selected for in vitro incubation. De Silva (1993) reported in a human IVF program, preparation and quality control of the embryo culture medium can be very time-consuming and expensive. Many
researchers believe that medium stored for up to 2 weeks is the limit, after that the medium is no longer suitable for embryo culture (Purdy, 1982; Quinn et al., 1984; Gerrity et al., 1992; McLendon, 1992).

There have been a few researchers who have shown that embryo culture medium can be stored or frozen for various amounts of time much longer than 2 weeks (Naz et al., 1986; Bernart et al., 1990; De Silva, 1993; Bell et al., 1995). This extended storage time would create many advantages for analyzing and selecting the best culture medium for human embryos. More assays could be run on the same batch of medium exposing more gametes and embryos to the same batch of medium thus, being more time efficient and inexpensive using the same medium for a longer period of time. Freezing culture medium would offer more opportunity for these advantages to be utilized.

However, there are many unknowns and very little research done in freezing embryo culture medium. It has been proposed that freezing the commercial culture medium at -80°C would create less of a biochemical breakdown of the components, therefore, allowing for an improved developmental rate for IVF embryos cultured in frozen-thawed medium.

In this experiment, a control CR1aa IVF culture medium was used to confirm laboratory techniques and to evaluate embryo development obtained with a protocol that had been previously proven in our laboratory. When cold storing the stock CR1aa control medium at 4°C for 3 months, we have observed similar or increased morula and blastocyst development rates when compared with the results reported with IVF-derived bovine embryos by Rosenkrans and First (1994).

The replicates were performed simultaneously with the fresh and frozen-thawed IVF procedures of our study to ensure technique and quality control for our laboratory. A significant difference was noted in 8- to 16-cell embryo development among a few replicates, however, when evaluating blastocyst development no significant difference among replicates was detected in CR1aa control culture medium. When development was further analyzed to the early blastocyst and expanded blastocyst stages a significant difference was found among early blastocyst development rates but not among expanded blastocyst development rates.
Cleavage was evaluated and recorded at two culture intervals in the present experiment for the commercial culture medium; the number of 2- to 8-cell embryos on day 4 of culture and the number of 8- to 16-cells on day 4 of culture. When evaluating the control storage time period Fresh 0 weeks versus frozen-thawed commercial culture medium there was a significant increase in cleavage found in 2- to 8-cell embryos cultured in Fresh 1 week medium and Frozen 1 week medium.

In this experiment, when evaluating 8- to 16-cell development we found no significant decrease in development when comparing the fresh control (0 weeks) commercial culture medium to any of the stored or frozen (-80°C) commercial culture medium treatments (Fresh 1 week, Fresh 2 weeks, Frozen 0 weeks, Frozen 1 week and Frozen 2 weeks). We did remove a significant outlier in the Frozen 1 week storage time period. An anomaly in culture is assumed to have caused this significant decrease in 8- to 16-cell development for this one replicate. Around the time period for this replicate the laboratory was undergoing construction and the floors were being waxed (a strong odor was recorded), however, why this treatment group would be the only group of that replicate that suffered significantly from these adverse effects is unknown. In storage period time Fresh 2 weeks and Frozen 0 weeks 8- to 16-cell embryos development is actually slightly higher than that of the control Fresh 0 weeks. Although there was a pattern for development to be greater for the 2 week stored medium and the 0 weeks frozen-thawed medium than the fresh control medium, there was no significant difference between the storage times and the control. However, there was a significant increase in development when comparing 8- to 16-cell stage embryos cultured in fresh medium (0 weeks) as opposed to those embryos cultured in medium frozen at -80°C for 2 weeks. There is no explanation for the increased development as frozen storage time increased.

There was no significant difference observed in blastocyst development when comparing the control storage time period Fresh 0 weeks versus any of the other fresh storage time periods or frozen storage time periods. In a similar study, Bernart et al. (1990) obtained results, using a different frozen-thawed culture medium, in murine and human embryo culture that are in agreement with those in our experiment. When Bernart et al. (1990) evaluated mouse embryos in Ham’s F-10 medium frozen at -20°C for up to 6 months there was no significant difference in embryo development. They then
evaluated the development of human embryos and found no significant difference in the rates of expanded and hatched blastocysts cultured in fresh Ham’s F-10 medium or in medium stored frozen for various intervals from 2 weeks to 3 months. As noted by Bernart et al. (1990) with human embryo culture, in our present study we saw a pattern of development to be greater for embryos cultured in medium frozen for 2 weeks compared with the control Fresh 0 week embryos. There was no significant difference noted in development when comparing different storage time periods of the frozen medium among each other.

When development was evaluated further to the early blastocyst and expanded blastocyst stages there was no difference in either developmental stage when compared among treatment groups. Bernart et al. (1990) found similar result when comparing development among murine embryo subgroups cultured in fresh or frozen medium for various intervals between 2 weeks and 6 months, there was no significant difference in the rates of expanded and hatched blastocyst stage embryos.

When examining mean RED scores of early blastocysts in the present study, there was no significant difference in the grades of IVF-derived bovine embryos among the control fresh storage time period versus any of the other fresh or frozen-thawed storage period times. There was no significant difference in mean RED scores among expanded blastocysts when evaluating the control fresh storage time period versus any of the other fresh or frozen-thawed storage period times. In contrast, Ryan et al. (1992) noted a significant effect of time in co-culture and treatment on resulting RED scores when analyzing heat stressed bovine embryos.

The results of this study indicate that freezing human commercial culture medium at -80°C (for 1 month), then storing for up to 2 weeks resulted in no significant decrease in 8- to 16-cell development or blastocyst development. The results of this study could be helpful to human fertility clinics in the future use of frozen-thawed culture media and the subsequent effects it may or may not have on embryo development at the 8- to 16-cell stage and blastocyst stage in vitro. Edwards et al. (1980) and Steptoe et al. (1980) first described the details of transferring cleavage stage embryos into the synchronized uterus of the human female. Since these early reports, it has been the norm of human fertility clinics to transfer embryos at the 8- to 16-cell stage. However, recent advances in culture media preparations have allowed cleavage stage human embryos to be
cultured out to the blastocyst stage (Barnes et al., 1995; Gardner et al., 1998; Jones and Trounson, 1999; Cruz et al., 1999; Behr et al., 1999; Schoolcraft et al., 1999; Milki et al., 2000; Gardner et al., 2000; Alper et al., 2001;). There are a number of advantages to culturing human embryos out to the blastocyst stage such as; selection for a higher quality embryo, reduced number of embryos transferred and decreased incidence of multiple gestation. Although there are advantages, as of today there are more research studies reported on transferring human embryos at the 8- to 16-cell stage. Clinicians are advised to evaluate data carefully before offering blastocyst transfer routinely, while we await an improved understanding of embryo development (Alper et al., 2001).

When analyzing the rest of the results development is not different in blastocyst numbers or RED score values indicating that overall embryos were not impaired in the commercial culture medium frozen for 1 month at -80°C and stored for up to 2 weeks. We can assume that GX2 commercial culture medium can be frozen at -80°C, stored for up to 2 weeks and used without any deleterious effects on day 8 bovine blastocyst development and morphology.

The findings of this study are promising to the future of culturing human embryos in frozen-thawed (-80°C) medium. Barnes and Eyestone (1990) indicated that although the media requirements are slightly different, similar mechanisms are observed in bovine and human embryo development. Brackett et al. (1982) stated that the further development and application of in vitro fertilization in cattle should provide information helpful in better management of problems related to human fertility and in efforts to develop procedures for human fertilization in vitro. There are a number of similarities in the human and bovine species a few being; similar gestation periods, both bearing a single offspring, as well as, the desirability for transfer of in vitro fertilized embryos into surrogates other than the ovum donor. Continued research of the bovine model is supported by these similarities between the human and bovine species and the findings of this study.
CHAPTER V
SUMMARY AND CONCLUSIONS

The primary objective of this study was to examine the potential of using frozen-thawed human commercial culture medium for successful IVF procedures. Little research has been reported on freezing human culture media and subsequent embryo post thaw development. New information on frozen-thawed culture media would allow fertility clinics to explore more efficient methods for IVF procedures by setting up improved quality control culture measures.

In this study, bovine embryos were used because it is illegal in most states to perform research on human embryos. The first experiment evaluated the in vitro development of bovine embryos in human commercial culture medium frozen and stored at -20°C. The medium (GX2) was frozen and stored for a period of 3 days to 10 weeks (Frozen 3 days, Frozen 2 weeks, Frozen 4 weeks, Frozen 6 weeks, Frozen 8 weeks and Frozen 10 weeks). Embryos were analyzed for development and morphology on day 4 and day 8 of in vitro culture. There was a significant decrease in development of the 8- to 16-cell stage embryos cultured in frozen medium for 8 to 10 weeks at -20°C. There was, however, no decrease in blastocyst development or embryo quality for the embryos cultured in medium frozen for up to 10 weeks at 20°C. There was no decrease in number of cell per embryo when blastocysts were cultured in medium frozen at -20°C for up to 10 weeks, when compared with the fresh control (Fresh 0 weeks).

In the second experiment, bovine embryos were used to evaluate human commercial culture medium frozen at -80°C (for 1 month). Again, there were a number of different fresh and frozen storage time periods utilized to culture these bovine embryos (Fresh 0 weeks, Fresh 1 week, Fresh 2 weeks, Frozen 0 weeks, Frozen 1 week and Frozen 2 weeks). The time periods in this experiment consisted of commercial culture medium being stored at 4°C from 0 to 2 weeks and frozen at -80°C for 1 month then stored at 4°C from 0 to 2 weeks. Embryos were analyzed for development and morphology on day 4 and day 8 of in vitro culture. The results indicate that 8- to 16-cell stage embryos cultured in medium frozen at -80°C and stored for up to 2 weeks resulted in no decreased development. Embryos reaching the 8-to 16-cell stage in frozen
medium (-80°C) and stored for 2 weeks actually showed a significant increase in development. No explanation can be made for this increased development in medium that was frozen/stored for 2 weeks over that of the control medium (Fresh 0 weeks). Overall, it was concluded that bovine embryos can be cultured in medium frozen at -80°C resulting in no decreased development or quality at the 8- to 16-cell stage or the blastocyst stage.

It was concluded that it is possible to freeze human commercial culture medium at either -20°C or -80°C without decreasing blastocyst development and overall embryo quality. To our knowledge this is the first report to evaluate the use of bovine embryos developed to the blastocyst stage (day 8) in frozen-thawed human commercial culture medium. The data and protocols gained herein can be used as guidelines to generate the next experiment or adapted as a model for fertility clinics trying to freeze/store embryo culture media.
LITERATURE CITED


Betteridge, K. J. and J. E. Flechon. 1988. The anatomy and physiology of pre-


Kovacic, B. V. Vlaisavljevic, M. Relijic, and V. Gavric Lovrec. 2002. Clinical outcome of day 2 versus day 5 transfer in cycles with one or two developed embryos. Fertil. Steril. 77:529-536.


Van den Berg, L. and D. Rose. 1959. Effect of freezing on the pH and composition of sodium and potassium phosphate solutions; The reciprocal system KH$_2$PO$_4$;Na$_2$HPO$_4$;H$_2$O. Arch. Biochem. Biophys. 81:319-329.


APPENDIX A: OOCYTE PROTOCOL AND MEDIA

Oocyte Aspiration Protocol (BOMED, Inc., Madison, WI)
1. Aspirate using a vacuum pump set a 5 psi into 50 ml conical tubes.
2. A stopper that fits the tube has two 18 g needles stuck through it, and a bent glass tube with the cut off end of a 1 ml syringe fitted to it so it can hold an 18 g needle.
3. Tygon tubing runs from the vacuum pump and also has a cut off 1 ml syringe attached to fit in one of the 18 g needles that runs through the stopper.
4. Follicles 2 mm or greater are aspirated until about 8 mm is obtained and the tube is filled.
5. Let sit 15 minutes and take out the settled pellet with a Pasteur.
6. Dilute with wash and search for oocytes.
7. Wash the pellet through a 70 µm tissue filter.

Oocyte Wash Medium (BOMED, Inc., Madison, WI)

<table>
<thead>
<tr>
<th>Component</th>
<th>Information</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL-HEPES</td>
<td>AB Technologies</td>
<td>-</td>
</tr>
<tr>
<td>BSA-Fraction-V</td>
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<td>3 mg/ml</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>Sigma</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Na pyruvate</td>
<td>Sigma</td>
<td>0.22 mM</td>
</tr>
</tbody>
</table>

Oocyte Maturation Medium (BOMED, Inc., Madison, WI)

<table>
<thead>
<tr>
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<th>Information</th>
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</tr>
</thead>
<tbody>
<tr>
<td>TCM199 with Earl’s salts</td>
<td>Cambrex</td>
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</tr>
<tr>
<td>10% FCS</td>
<td>Cambrex</td>
<td>-</td>
</tr>
<tr>
<td>Bovine LH</td>
<td>Sioux Biochemicals</td>
<td>3 µg/ml</td>
</tr>
<tr>
<td>Bovine FSH</td>
<td>Sioux Biochemicals</td>
<td>3 µg/ml</td>
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<tr>
<td>Gentamycin</td>
<td>Sigma</td>
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</tr>
<tr>
<td>Na pyruvate</td>
<td>Sigma</td>
<td>0.22 mM</td>
</tr>
</tbody>
</table>

Filter with Millipore filters and put into vials, equilibrate in incubator for several hours.
APPENDIX B: FERTILIZATION MEDIUM, PROTOCOL AND CR1aa STOCK MEDIUM

Oocyte Fertilization Medium (BOMED, Inc., Madison, WI)

Supplementation of IVF (Specialty Media Inc., Lavallette, NJ)

<table>
<thead>
<tr>
<th>Component</th>
<th>Information</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid-free BSA</td>
<td>-</td>
<td>6 mg/ml</td>
</tr>
<tr>
<td>Gentamycin stock (50 mg/ml)</td>
<td>Sigma or Cambrex</td>
<td>0.5 µg/ml</td>
</tr>
<tr>
<td>Na pyruvate stock</td>
<td>-</td>
<td>10 µg/ml</td>
</tr>
</tbody>
</table>

Na pyruvate stock made fresh daily (22 mg/5 ml of TL-HEPES). Millipore with 0.22 µm filter and allow to equilibrate 3 to 4 hours in incubator.

Fertilization Protocol (BOMED, Inc., Madison, WI)

1. Gas mix is 5% CO₂ in air.
2. Sperm/oocyte culture is done 18 to 20 h at 39°C in high humidity.
3. PHE and heparin are used as capacitation agents.
4. Sperm is prepared for fertilization through 45/90% Percoll gradient.
5. Sperm are then prepped in TL-HEPES wash.
6. Sperm are used at 1 x 10⁶ per ml final concentration.
7. Oocyte complexes and sperm are co-incubated for 16 to 20 hours.
8. Oocyte complexes are then vortexed and washed in TL-HEPES wash and placed into SOFaa for culture or TL-HEPES with BSA for shipping.

CR1aa Stock Medium (100 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Information</th>
<th>Amount</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>S-5886 (Sigma)</td>
<td>0.6703 g</td>
</tr>
<tr>
<td>KCl</td>
<td>S-5405 (Sigma)</td>
<td>0.0231 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>S-8875 (Sigma)</td>
<td>0.2201 g</td>
</tr>
<tr>
<td>Pyruvic Acid</td>
<td>P-2256 or P-4562 (Sigma)</td>
<td>0.0044 g</td>
</tr>
<tr>
<td>L(+)Lactic Acid</td>
<td>L-4388 (Sigma)</td>
<td>0.0546 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>G-8790 (Sigma)</td>
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</tr>
<tr>
<td>L-Alanine</td>
<td>A-7469 (Sigma)</td>
<td>0.0045 g</td>
</tr>
</tbody>
</table>
APPENDIX C: CR1aa CULTURE MEDIUM

Milli Q water (DD water) - 100 ml
Phenol Red 0.5% P-0290 0.2 ml (200 µl)

Make in a 100 ml bottle and then filter into two 50 ml tubes.
Place para film around the bottle and store in refrigerator for up to 3 months.

CR1aa Embryo Culture Medium for Day 1 Through Day 4 of Culture (10 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Information</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1aa Stock</td>
<td>-</td>
<td>9.5 ml</td>
</tr>
<tr>
<td>BME aa solution (50X)</td>
<td>B-6766 (Sigma)</td>
<td>200 µl</td>
</tr>
<tr>
<td>MEM aa solution (10X)</td>
<td>11140-050 (Gibco)</td>
<td>100 µl</td>
</tr>
<tr>
<td>Gentamycin (50 mg/ml)</td>
<td>15750-060 (Gibco)</td>
<td>10 µl</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>G-5763 (Sigma)</td>
<td>0.00146 g</td>
</tr>
<tr>
<td>BSA</td>
<td>A-7511 Sigma</td>
<td>0.03 g</td>
</tr>
<tr>
<td>Insulin</td>
<td>-</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Make in 15 ml conical tube, filter and place in incubator for 30 to 45 minutes.

CR1aa Embryo Culture Medium for Day 4 Through Day 8 of Culture (10 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Information</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1aa Stock</td>
<td>-</td>
<td>9.5 ml</td>
</tr>
<tr>
<td>BME aa solution (50X)</td>
<td>B-6766 (Sigma)</td>
<td>200 µl</td>
</tr>
<tr>
<td>MEM aa solution (100X)</td>
<td>11140-050 (Gibco)</td>
<td>100 µl</td>
</tr>
<tr>
<td>FBS (Fetal bovine serum)</td>
<td>-</td>
<td>500 µl</td>
</tr>
<tr>
<td>Gentamycin (50 mg/ml)</td>
<td>15750-060 (Gibco)</td>
<td>10 µl</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>G-5763 (Sigma)</td>
<td>0.00146 g</td>
</tr>
<tr>
<td>BSA</td>
<td>A-7511 Sigma</td>
<td>0.03 g</td>
</tr>
<tr>
<td>Insulin</td>
<td>-</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Make in 15 ml conical tube, filter and place in incubator for 30 to 45 minutes.
## APPENDIX D: EXPERIMENT I WEEKLY REPLICATES

Appendix D. Six weekly replicates performed from April 30\(^{th}\) to July 9\(^{th}\) containing a group of bovine zygotes in vitro cultured in each treatment media.

<table>
<thead>
<tr>
<th>Replicate Number</th>
<th>Replicate Date</th>
<th>Media Used</th>
<th>Number of zygotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/30/2004</td>
<td>CR1aa</td>
<td>30</td>
</tr>
<tr>
<td>1</td>
<td>4/30/2004</td>
<td>Fresh 0 weeks</td>
<td>30</td>
</tr>
<tr>
<td>1</td>
<td>4/30/2004</td>
<td>Frozen 10 weeks</td>
<td>130</td>
</tr>
<tr>
<td>1</td>
<td>4/30/2004</td>
<td>Frozen 2 weeks</td>
<td>130</td>
</tr>
<tr>
<td>1</td>
<td>4/30/2004</td>
<td>Frozen 3 days</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>5/14/2004</td>
<td>CR1aa</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>5/14/2004</td>
<td>Fresh 0 weeks</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>5/14/2004</td>
<td>Frozen 4 weeks</td>
<td>130</td>
</tr>
<tr>
<td>2</td>
<td>5/14/2004</td>
<td>Frozen 2 weeks</td>
<td>130</td>
</tr>
<tr>
<td>2</td>
<td>5/14/2004</td>
<td>Frozen 3 days</td>
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</tr>
<tr>
<td>3</td>
<td>5/28/2004</td>
<td>CR1aa</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>5/28/2004</td>
<td>Fresh 0 weeks</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>5/28/2004</td>
<td>Frozen 6 weeks</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>5/28/2004</td>
<td>Frozen 4 weeks</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>5/28/2004</td>
<td>Frozen 2 weeks</td>
<td>96</td>
</tr>
<tr>
<td>4</td>
<td>6/11/2004</td>
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<td>30</td>
</tr>
<tr>
<td>4</td>
<td>6/11/2004</td>
<td>Fresh 0 weeks</td>
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</tr>
<tr>
<td>4</td>
<td>6/11/2004</td>
<td>Frozen 8 weeks</td>
<td>96</td>
</tr>
<tr>
<td>4</td>
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<td>Frozen 6 weeks</td>
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<tr>
<td>4</td>
<td>6/11/2004</td>
<td>Frozen 4 weeks</td>
<td>96</td>
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<tr>
<td>5</td>
<td>6/25/2004</td>
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</tr>
<tr>
<td>5</td>
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<td>30</td>
</tr>
<tr>
<td>5</td>
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<td>Frozen 10 weeks</td>
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<td>6/25/2004</td>
<td>Frozen 8 weeks</td>
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<tr>
<td>5</td>
<td>6/25/2004</td>
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<td>96</td>
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<tr>
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<td>7/9/2004</td>
<td>Fresh 0 weeks</td>
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<td>7/9/2004</td>
<td>Frozen 10 weeks</td>
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</tr>
<tr>
<td>6</td>
<td>7/9/2004</td>
<td>Frozen 8 weeks</td>
<td>130</td>
</tr>
<tr>
<td>6</td>
<td>7/9/2004</td>
<td>Frozen 3 days</td>
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</tr>
</tbody>
</table>
APPENDIX E: EXPERIMENT II WEEKLY REPLICATES

Appendix E. Twelve weekly replicates performed from January 13th to May 5th containing a group of bovine zygotes in vitro cultured in each treatment media.

<table>
<thead>
<tr>
<th>Replicate Number</th>
<th>Replicate Date</th>
<th>Media Used</th>
<th>Number of zygotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/13/2005</td>
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<td>50</td>
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<tr>
<td>1</td>
<td>1/13/2005</td>
<td>Fresh 0 weeks</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>1/13/2005</td>
<td>Fresh 2 weeks</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>1/13/2005</td>
<td>Frozen 0 weeks</td>
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</tr>
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<td>3</td>
<td>1/27/2005</td>
<td>CR1aa</td>
<td>96</td>
</tr>
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<td>1/27/2005</td>
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<td>100</td>
</tr>
<tr>
<td>3</td>
<td>1/27/2005</td>
<td>Frozen 2 weeks</td>
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<td>Fresh 2 weeks</td>
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VITA

Jeanne Lee Glaser was born in Baton Rouge, Louisiana, to Jaqueline C. Martin and Theodore H. Glaser III. Jeanne has a younger brother, Ryan Jude Glaser, who graduated in the fall of 2006 with his bachelor degree in agricultural economics from Louisiana State University, Baton Rouge, and a younger sister Kimberly R. Glaser, who is currently attending Lake Sherwood Academy. Jeanne grew up in Maringouin and Oscar, Louisiana, while she attended the Catholic Interparochial school, Catholic High School of Pointe Coupee in New Roads, Louisiana. Following graduation in 1999, Jeanne attended Northwestern State University, Natchitoches, Louisiana, from the fall of 1999 to the spring of 2000. In the fall of 2000 Jeanne enrolled at Louisiana State University, Baton Rouge, Louisiana. After taking an enthusing undergraduate class her senior year with Dr. Robert A. Godke, Jeanne received her bachelor degree in animal science in the fall of 2003. In August 2004, Jeanne began to pursue her Master of Science degree under the guidance of Dr. Robert A. Godke, Boyd Professor of Reproductive Physiology. She is a candidate for the Master of Science degree in reproductive physiology in the Department of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.