

Improving the Development of Bovine In Vitro Produced Embryos Cultured Individually

Bethany Gale Gibson

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Biomedical and Veterinary Sciences

Sherrie G. Clark-Deener

Willard H. Eyestone

Alan D. Ealy

June 26, 2014

Blacksburg, VA

Keywords: bovine, embryo, individual culture

Copyright 2014 Bethany G. Gibson

Improving the Development of Bovine In Vitro Produced Embryos Cultured Individually

Bethany G. Gibson

Abstract

Previous research in bovine embryology has found that embryos cultured individually have limited ability to develop compared to their counterparts cultured in a group of other embryos. This investigation aimed to find if any of three different interventions over two experiments would increase development of individually cultured embryos to that of group cultured embryos. In the first experiment both the addition of serum/serum replacer and a co-culture with bovine granulosa cells were applied to individually cultured embryos in a 3x2 design. None of the interventions was found to be significantly different from the others, and all resulted in significantly lower development than embryos cultured as a group (avg. 4.7 +/- 1.93% individual vs. 21.7 +/- 3.76% group). However, a significant difference was found in the hatching rate between blastocysts cultured in media including cells (71.4 +/- 17.07%) and those cultured without cells (18.1 +/- 11.63%). In the second experiment, embryos were either cultured in standard droplets or microwells made at the bottom of culture droplets either in groups or individually for a 2x2 design. This experiment experienced poor development in all treatments including the group control, and none of the treatments were found to be significantly different from each other. However, the hatching rate of blastocysts cultured in multiple microwells was significantly higher than those cultured individually in droplets. To summarize, none of the treatments increased the development rate, but embryos cultured with granulosa cell co-cultures and in group microwells showed improvements in hatching rates.

Acknowledgements

I would like to thank:

my advisor, Dr. Sherrie Clark-Deener for the encouragement and funding to do this project as well as the opportunity to continue my education here at VMRCVM. Dr. Julie Cecere for tirelessly teaching me through the ups and downs of clinical theriogenology. Dr. Will Eyestone for his patiently teaching me the ins and outs of in vitro embryo production, as well as helping with study design. And Dr. Alan Ealy was instrumental in providing ideas and input for both projects.

those that assisted in large and small ways in this research: Maria Cristina Villafranca Locher, DVM, DVM students Emma Corrigan and Ryan Giles, Russell Harris and the entire staff of Brown Packing Co. in Gaffney, SC.

the statistical people who worked with me to accurately report the results of my work – Dr. Michael McGilliard, Dr. François Elvinger, and especially Dr. Stephen Werre.

my family for their unwavering support, Sarah Holland and my fellow resident-mates at VMRCVM, and my church family at Northstar Church in Blacksburg.

And most of all, God, for leading me on such an unpredictably amazing path.

Source of Funding

The funding for this project was provided by the Virginia-Maryland Regional College of Veterinary Medicine as part of start-up research funds provided to my advisor, Dr. Sherrie Clark-Deener. I will be forever indebted to the commitment to research shown by the VMRCVM as well as the generosity of Dr. Clark-Deener to share what was provided to her.

Table of Contents

Abstract	ii
Acknowledgements	iii
Source of Funding	iv
List of Figures	vii
List of Tables	viii
Chapter 1: Literature Review	1
Rationale for Project.....	1
History of In-Vitro Embryo Production	1
In vivo maturation	2
In vitro maturation.....	3
In vivo fertilization.....	6
In vitro fertilization.....	7
In vivo embryo culture and early embryo development.....	8
In vitro embryo culture	9
Cellular Co-culture	12
Serum Added to Culture Media.....	14
Modern Media Choices	16
Assessment of In Vitro Produced Embryo Quality and Health.....	17
Individual Embryo Culture.....	18
Media Additives for Single Embryo Culture.....	19
Physical Changes to the Culture Environment	21
Importance of In Vitro Production to the cattle industry.....	24
Chapter 2: Manuscript	25
Abstract	25
Introduction	26
Materials and Methods	29
Experiment 1	29
Preparation of the culture environment	29
In vitro embryo production.....	30
Differential staining procedure.....	31

Experiment 2	32
Preparation of well-of-the-well culture dishes	32
In vitro embryo production.....	33
Statistical analysis	33
Results	33
Discussion	35
Low individual embryo development.....	37
Chapter 3: Conclusion	42
References	56
Appendix A – Media Formulations	66
Appendix B – Permission Letters.....	71

List of Figures

Figure 1. Illustration of the Well-of-the-Well System compared to the traditional culture drop system.....	43
Figure 2. Experiment 1: Rate of Development to Blastocyst per Oocyte by Treatment.	47
Figure 3. Experiment 1: Development Rate Pooled by Presence/Absence of Granulosa Cells Independent of Media Additive.....	48
Figure 4. Experiment 1: Development Rate Pooled by Media Additive Independent of Presence or Absence of Granulosa Cell Co-Culture.....	49
Figure 5. Experiment 1: Hatching Rate by Treatment.....	50
Figure 6. Experiment 1: Hatching Rate Pooled by Presence/Absence of Granulosa Cells Independent of Media Additive.....	51
Figure 7. Experiment 1: Inner Cell Mass Percentage by Treatment.....	52
Figure 8. Experiment 1: Inner Cell Mass Percentage Pooled by Presence/Absence of Cells Independent of Media Additive.....	53
Figure 9. Experiment 2: Development Rate to Blastocyst per Oocyte by Treatment.	54
Figure 10. Experiment 2: Hatching Rate by Treatment.....	55

List of Tables

Table 1. Individual Culture Treatment Conditions in Experiment 1.....	44
Table 2. Treatment Conditions in Experiment 2.	45
Table 3. Number of oocytes per group in Experiment 1: Comparison of additives (FBS, SR or no additive) in a culture system with or without granulosa cell co-culture.....	46
Table A-1. Cell Culture Medium.....	66
Table A-2. Embryo Culture Medium.	67
Table A-3. SOF-HEPES.....	68
Table A-4. Maturation Medium.	69
Table A-5. Fertilization Medium.....	70

Chapter 1: Literature Review

Rationale for Project

In research as well as clinical practice, in vitro produced bovine embryos are cultured in groups. Developing an individual culture system has several potential benefits (Goovaerts *et al.* 2010). First, it will benefit commercial in vitro embryo production in cases of low oocyte recovery during ovum pick-up or postmortem oocyte recovery procedures, and in cases where individual identification of embryos is necessary (sex determination, genomic testing, etc). Second, it will assist in research when investigators want to draw conclusions about the relative competence of oocytes from dams of specific metabolic states as the relative impact of oocyte competence and early uterine environment continues to be studied for purposes of determining the causes (or likely causes) of lower fertility in high producing cows. As well, it could provide a more consistent culture environment which does not depend on the factors secreted by other embryos. For all of these reasons a reliable system of individual bovine embryo culture is desirable.

History of In-Vitro Embryo Production

In-vitro embryo production involves 3 steps: maturation of oocytes, fertilization with motile, capacitated sperm, and then culture to the blastocyst stage. It is still almost unfathomable that it is actually possible to begin with an immature oocyte and end with a developed embryo outside of an animal. Thus, the researchers of today owe a huge debt to the researchers of yesterday, as there were many, many investigations and failed attempts which laid the foundation for the eventual successes found in the 1980s and still enjoyed today.

In vivo maturation

Before exploring in vitro maturation, it is prudent to consider the details of maturation in vivo. Follicles are stimulated to change from primordial to primary follicles under the influence of as-yet unknown signals. Once they become primary follicles, they develop a zona pellucida and experience abundant hyperplasia of the surrounding granulosa cells as they become a preantral follicle over a period of about 3-4 months (Scaramuzzi *et al.* 2011). At this point, they are considered gonadotropin-responsive and will continue to grow and develop under the influence of follicle stimulating hormone (FSH) secreted by the anterior pituitary in response to gonadotropin releasing hormone (GnRH) secreted by the hypothalamus. As the preantral follicles respond to FSH, one or more will grow larger than the others and become the dominant follicle. As the follicle grows (due mostly to increasing antrum size, although the oocyte itself also approximately quadruples in size), it becomes dependent on FSH for its final maturation and development. Just before ovulation the majority of the hormone receptors present on the dominant follicle will change from FSH to luteinizing hormone (LH) receptors, which occurs in concert with falling FSH concentrations (due to production of inhibin by the dominant follicle). This assures dominance of the follicle, as other, less developed follicles do not have this changeover in receptors and are still FSH dependent (Scaramuzzi *et al.* 2011). The final step in in vivo maturation occurs in response to the LH surge, which precedes ovulation by approximately 24 hours. During this pre-ovulatory maturation (sometimes called oocyte capacitation) the oocyte resumes meiosis to arrest at the metaphase II step. The granulosa cells which are in direct contact with the oocyte (called corona radiata cells) have had very intimate contact with the cytoplasm of the oocyte through zona processes which pass through the zona pellucida and are connected to the oocyte through gap junctions. These gap junctions allow the passage of several cytoplasmic molecules, including cyclic adenosine monophosphate (cAMP).

Prior to the LH surge, those processes retreat somewhat from the cytoplasm, and after the surge they retract more until they are completely removed from the oocyte (Dieleman *et al.* 2002). This loss of communication causes, among other things, a decrease in cAMP in the oocyte. This falling level of cAMP within the oocyte is suspected to be a signal for resumption of meiosis (Eppig 1989).

In vitro maturation

The goal of in vitro maturation (IVM) is to cause an immature oocyte from a developing follicle to resume meiosis and render itself capable of fertilization, arrested at the second metaphase step of meiosis just like its in vivo matured counterpart. Early researchers (Hunter *et al.* 1972) found that oocyte maturation (as indicated by nuclear changes in chromatin and germinal vesicle breakdown (GVBD)) seemed to proceed spontaneously when oocytes were removed from the follicle. The mechanisms of this spontaneous resumption of meiosis, however, were not entirely clear at that time. Downs and Eppig (1984) found that culturing murine oocytes in follicular fluid caused a transient suspension of maturation (signaled by a prevention of GVBD), and further, the addition of a cAMP elevating agent ((Bu)₂cAMP) inhibited maturation for at least 21 hours (compared to nearly 100% GVBD after 3 hours in controls). Thus, it seems that the necessary steps to initiate the maturation process in vitro include both removal of the oocyte from the follicular environment and depletion of intracellular cAMP stores. The first report of successful IVM (indicated by polar body extrusion and metaphase plate appearance) of a bovine oocyte was by Edwards (1965). The major medium components (cell culture medium, serum, and antibiotics) and incubation conditions (5% CO₂ in humidified air) described in this report are, with only slight modifications, still used today. Soon after, similar work was conducted by another group showing that oocytes could start the maturation process in vitro (to

metaphase I), continue in vivo, and be successfully fertilized in vivo (shown by two pronuclei or evidence of sperm within the ooplasm) (Hunter *et al.* 1972). The first report of a blastocyst produced in vivo after in vitro maturation (in fetal bovine serum (FBS) alone) was completed by Trounson *et al.* (1977). Leibfried-Rutledge *et al.* (1987) performed IVM in a media very similar to Edwards with the addition of pyruvate as well as porcine-origin follicle stimulating hormone (FSH). That study showed that although 39% of in-vitro matured oocytes appeared to undergo normal fertilization after IVF (compared to 71% of in vivo matured, in vitro fertilized controls), there were no blastocysts recovered after in vivo culture (compared to 37% of controls). This showed that in vitro matured oocytes were still quite hampered compared to their in vivo matured counterparts, and despite being capable of experiencing apparently normal fertilization, further development was severely depressed. A study published one year later from the same researchers (Sirard *et al.* 1988) showed vastly improved results – the biggest difference in the IVM protocol was the substitution of purified and defined FSH, as well as the addition of purified and defined LH and estradiol. Their results showed improved development over previous work, as 28% of cleaved embryos recovered after in vivo culture had proceeded to the morula or blastocyst stage. No control in vivo matured group was included, but a group of oocytes subjected to IVM in media devoid of hormones resulted in a development rate of 18% which was not significantly different than the hormone-included group. The inclusion of the gonadotropins is intended to mimic the in vivo environment, and both LH (Zuelke and Brackett 1990) and FSH (Caixeta *et al.* 2013) have shown to improve the health of the oocyte or surrounding cumulus cells. Steroid hormones present a bit of an enigma, as the granulosa cells which surround the oocyte have been shown to secrete estrogen, testosterone, and progesterone during IVM (Schoenfelder *et al.* 2003). Although they may not be added to the media, they are likely present in unmeasured amounts. However, Clemente *et al.* (2009) found that progesterone concentrations declined precipitously in media which was in contact with mineral oil (vs. no change in media not in contact with oil).

These studies suggest that concentrations of steroids are rather undefined in the media and may be very sensitive to environmental conditions.

The results some of the early IVM studies (i.e. Trounson *et al.* 1977; Leibfried-Rutledge *et al.* 1987; Sirard *et al.* 1988) suggest that FBS alone is a media capable of supporting oocyte maturation. However, as the negative effects of serum (discussed later) were discovered, many researchers changed to using FBS only in small concentrations or excluding it altogether from maturation media. Instead, cell culture media such as TCM 199 or DMEM often make up the majority of maturation media. In the pursuit of more defined maturation media, Rose-Hellekant *et al.* (1998) found that of several serum free maturation media tested, the common factor among those which produced the highest percentage of blastocysts after IVF and in vitro culture (IVC) was glucose supplementation. A further investigation (Hashimoto *et al.* 2000) confirmed that moderate (1.5 mM) glucose supplementation in IVM media augments blastocyst development (compared to no supplementation) but revealed that an excess (20 mM) leads to comparatively depressed development after IVF/IVC. This depression in development was seen in spite of the fact that more of the oocytes matured in the high glucose group showed nuclear evidence of progression to metaphase II than those in the moderate glucose media, and fertilization between the two groups appeared to be equal. Other additives which have shown benefit when added to maturation media are the gonadotropins (LH and FSH). IVM has been successfully carried out successfully in defined media without the addition of gonadotropins (i.e. Rose-Hellekant *et al.* 1998), however, many media used in IVM today include gonadotropins as the eventual development of oocytes matured in their presence following in vitro production appears to be higher than those matured without gonadotropins (Rodriguez and Farin 2004).

In vivo fertilization

In vivo fertilization in the bovine takes place in the reproductive tract of the female when male and female gametes meet and combine. Normally, sperm is deposited just outside the cervix (natural service) or just inside the cervix (artificial insemination). Spermatozoa unite with oocytes in the oviduct, the tube connecting ovary to uterus. According to Holt et al. (2010), this relatively long journey through the uterus serves to select for the fastest swimming sperm, which are often those with the longest flagella. However, it is not simply a matter of sperm meeting egg – much like the oocyte (discussed previously), sperm must undergo capacitation in order to successfully fertilize. This became clear when Chang (1951) proved in a rabbit model that sperm needed time in the female tract to acquire the ability to successfully fertilize an oocyte. Capacitation in sperm means that it is prepared to undergo the acrosome reaction when it meets the zona pellucida. It involves stripping many of the seminal plasma proteins from the head of the sperm which were attached during ejaculation. Much of what is known about sperm capacitation comes from studies of sperm in vitro (discussed later). In vitro studies (Parrish 2014) have shown that capacitation takes 4-6 hours, but these time points are not as well defined in vivo due to many factors specific to the uterine environment (Rodriguez-Martinez 2007). The aforementioned acrosome reaction involves fusion of the plasma and acrosomal membranes of the sperm head, which allows proteolytic enzymes to leave the sperm head to help break down a small area of the the zona pellucida to assist with sperm entry into the perivitelline space. The entry of a single sperm initiates a cascade of events inside the oocyte leading to the zona block which should prevent any other sperm from penetrating. This is important because an oocyte which is fertilized by more than one sperm (a phenomenon called polyspermy) is nonviable.

In vitro fertilization

In vitro fertilization (IVF) showed itself as an equal if not greater challenge than IVM, likely owing to the fact that conditions needed to be optimized for two gametes instead of one. The foundational work can again be found reviewed in Wright and Bondioli (1981). After completing the aforementioned in vivo capacitation work, Chang went on to carry out one of the first studies to demonstrate successful capacitation which involved flushing sperm from the uterus and oviducts of a rabbit bred 18h previously to accomplish a low but meaningful rate of IVF (1959). This system of capacitation, although successful, was not practical. Brackett *et al.* (1978) developed a high ionic strength system of incubation which induced spermatocapacitation in vitro and resulted in sperm capable of successfully fertilizing rabbit ova. Just a few years later, he described the birth of the first calf as a result of in vitro fertilization (Brackett *et al.* 1982). The oocytes were matured in vivo, collected from the oviduct of donor heifers, coincubated with approximately 1 million freshly collected sperm (which had previously been incubated in a high ionic strength solution for 3 hours) in 4 mL fertilization media, and the resulting embryos were transferred surgically to the recipient cows who had a natural estrus at the same time as the donor when the embryo was at the 4 cell stage. One live birth (Virgil) resulted. This method, although preliminarily successful, was difficult to replicate and appeared to only work for certain bulls (Parrish 2014).

Further investigations into the composition of oviductal fluid (which induces capacitation in vivo) showed very high levels of glycosaminoglycans (GAGs), and a new focus for elucidating the induction of capacitation was found. Parrish *et al.* (1985) found both that the GAG heparin sulfate significantly improved fertilization, as well as that this improvement was reduced by the presence of glucose in the media. Heparin binds to seminal plasma proteins present on the sperm head, eventually causing the number of these proteins to decrease. However, changes induced by

capacitation do not stop at the surface – indeed, the discovery of heparin as a capacitation agent has aided in the understanding of intracellular changes in the sperm due to capacitation. In the intracellular environment of the sperm, heparin induces alkalinization, increases the amount of cAMP present (Parrish 2014), and increases calcium concentrations both in the acrosome and the intracellular environment of the sperm head (Parrish *et al.* 1999). Glycolysis induced by the presence of glucose acidifies the intracellular environment (thus antagonizing the heparin-induced alkalinization), hence the reduction in capacitation in media including glucose. Since its discovery, heparin has become the de facto capacitation agent in bovine IVF.

In vivo embryo culture and early embryo development

In its natural environment, the developing embryo stays in the oviduct until 4-7 days post fertilization. The embryo is often at the morula or blastocyst stage by this point. Much of the work done in in vitro culture methods has shown that in vivo cultured control embryos hold advantages over their counterparts in vitro (Lonergan *et al.* 2003; Lonergan *et al.* 2006; Tesfaye *et al.* 2007).

The developing embryo (both in vivo and in vitro) goes through several recognizable stages of growth. A one-celled structure where male and female pronuclei have fused is called a zygote (Senger 2005). In the first few mitotic divisions, growth is only in cell number, as the original cytoplasm of the oocyte is divided among the daughter cells. Each blastomere possesses the quality of totipotency, meaning that it could be induced to become any fetal or adult cell type. After the fourth cell cycle (when the embryo is made up of 8 cells), the embryo undergoes the “maternal to embryonic transition” which means that the embryo is charged with the task of making its own mRNA instead of using the stockpiled maternal mRNA present in the oocyte.

The embryonic genome is highly methylated (and thus unavailable for transcription) in the gamete, so it must be both de- and re-methylated (reviewed in Zhao *et al.* 2010; Kepkova *et al.* 2011). Not surprisingly, this process is a critical obstacle which is difficult for embryos to overcome (see eight cell block, discussed later). After the blastomeres become too numerous to count (16 cell stage, at the earliest), the embryo is called a morula. Further, this is usually the time at which the cells become differentiated into cells which make up the inner cell mass and those of the trophectoderm. Thus, although they are still pluripotent, they are totipotent no longer. The inner cell mass gives rise to the embryo proper as well as other extraembryonic membranes, and the trophectoderm becomes the chorion, or the fetal contribution to the placenta. The final developmental milestone is signaled when the embryo develops a fluid-filled cavity called a blastocoel, around which primitive endoderm will enclose the yolk sac. It is at this, the blastocyst stage, when many in vivo or in vitro produced embryos in commercial production are evaluated and transferred to appropriately timed recipients.

In vitro embryo culture

In the in vitro production of embryos, two of the three pieces of the puzzle were in place. Obviously, work continued to improve methods of maturation and fertilization, but embryo culture stood as the final great frontier to in vitro embryo production. The benefits offered by IVC are easy to see, such as the ability to observe development in real time, as well as the ability to non-surgically transfer embryos at a later stage of development. The latter is absolutely essential for the widespread commercial application of in vitro embryo production. The early work in this area involving bovine embryos is reviewed in Wright and Bondioli (1981). Although each investigation provided some foundational framework for the investigators who came later,

there were a few discoveries which led to large leaps in our understanding of embryo culture, and they will be the focus of this discourse.

The first several attempts at bovine IVC used a variety of media. Dowling (1949) used a saline with egg white medium (which was first used by Hammond (1949) successfully in murine embryo culture). Dowling's research focused on superovulated in vivo matured and fertilized embryos collected at varying stages post fertilization. One donor whose embryos were collected 3.5 days post insemination gave 14 8-cell embryos. All of them divided once in 24 hours of culture in the egg-saline medium. Pincus (1951) attempted culture of in vivo produced embryos at varying stages of development in bovine serum. He observed less than 1 division per embryo after culture for 72 hours. Brock and Rowson (1952) attempted to culture in vivo produced embryos in follicular fluid but observed no further development. In the same experiment, 3 of 4 embryos cultured in bovine serum at the 4-cell stage divided once, but embryos cultured at other stages did not continue to develop.

Although the media choices of the initial attempts at in vitro culture were logical as they supplied protein, nutrients, and growth factors, embryo culture in them was relatively unrewarding. Researchers set out to find exactly what type of fluid embryos were cultured in in vivo. Restall and Wales (1966) were the first to elucidate the ionic and protein contents of the oviductal fluid of sheep. Appropriately, the resulting media was called synthetic oviductal fluid (SOF), and forms the basis for many ruminant in vitro production protocols of today. SOF was used for the first time in bovine embryo culture by Tervit *et al.* (1972). The in vivo produced embryos that started at the eight cell stage proceeded to the morula stage in 30% of embryos, which represented only the second time that a morula had been produced in culture. In the same study, oxygen tension was varied to better approximate the conditions of the oviduct (borrowing a page from murine embryo technology (Whitten 1971)). When O₂ levels were reduced to 5%

(from the 21% found in the atmosphere) with 5% carbon dioxide and the balance nitrogen, development of eight cell in vivo produced embryos proceeded appropriately and the world saw for the first time a bovine blastocyst as the result of in vitro culture. The lower oxygen tension is thought to reduce the load of reactive oxygen species present in the media (Noda *et al.* 1991). Despite early success (Tervit *et al.* 1972), low oxygen tension was not immediately adopted by all researchers of the time. However, after this relatively slow adoption, the large majority of embryo projects performed today have adopted the lower oxygen tension. In doing this, they have experienced similar successes in development.

Early successes in bovine embryo development were often seen either from 1-2 cell embryos to the eight cell stage, or from the eight cell stage to morulae/blastocyst. Thus, the term “8 cell block” was coined to describe the insufficiency of culture systems of the day to support development through the fourth cell cycle. Interestingly, this “block” occurs at the same point as embryonic genome activation (EGA), or the shift in the embryo from the embryo’s dependence on maternal mRNA to embryonic. The same pattern is seen in murine embryo development, where a similar “block” is commonly encountered at the two cell stage, which again is the point of EGA for that species (Telford *et al.* 1990). It is unclear if these two phenomena are related, but the pattern certainly raises suspicion of a correlation. The application of low oxygen tension in bovine embryo culture was the first step in solving the 8-cell block (Tervit *et al.* 1972). Although he was not the first to successfully bypass it (possibly owing to the omission of amino acids from the culture media (Gardner *et al.* 1994)), his work laid the foundation for others to come after him. Eyestone and First (1991) found that embryos in the third and fourth cell cycle were most affected by in vitro culture conditions, and would not proceed in development in vivo after in vitro culture for 24 hours, even though embryos cultured for short periods in vitro at other points in development could proceed to the blastocyst stage after in vivo culture. Vital staining

showed that this cessation of development was not due to embryo death, but a suspension in embryo development. In addition to low oxygen tension, somatic cell co-culture (discussed next) has also been shown to overcome this “block.”

Cellular Co-culture

In a page borrowed from murine embryo culture (Biggers *et al.* 1962), investigators discovered that somatic cell co-culture was beneficial to bovine IVC and helped to overcome the aforementioned “8 cell block.” One of the first bovine experiments to use this approach was carried out by Camous *et al.* (1984). In vivo produced embryos were cultured in media including serum with and without trophoblastic vesicles. Trophoblastic vesicles are made from in vivo produced day 14 bovine embryos which have had their inner cell mass removed. They have been shown to persist in in vitro culture for at least 5 days, and to maintain corpora lutea in vivo (Heyman *et al.* 1984). The results showed that the previously identified block to development was overcome in both treatment groups, but to a greater extent in the co-culture group (46% vs. 18% development to morula). These results, while encouraging, presented difficulties in replication as they depend on a constant fresh supply of bovine blastocysts because the trophoblast could only be maintained in culture for around 5 days. Another co-culture system had previously been reported to improve development of in vivo produced mouse embryos (Biggers *et al.* 1962). This study used whole-organ co-culture of the fallopian tube with embryos, and noted the ability of embryos to bypass the 2 cell block (analogous to the 8 cell block in the bovine). This makes sense, because if the cultured oviductal cells behave similarly in vitro as in vivo, they may be expected to approximate the in vivo environment by producing growth factors and removing wastes. Gandolfi and Moore (1987) found that culturing in vivo produced ovine embryos on an oviductal epithelial monolayer significantly improved development to the

blastocyst. Thus, Eyestone and First (1989) chose to focus on a co-culture of oviductal cells in bovine embryo culture. This was a seminal paper for two reasons: it was the first to show the beneficial effect of oviductal co-culture (or oviductal conditioned media) on bovine embryo development, and it was the first to demonstrate a fully in vitro system of embryo production which resulted in the birth of 5 normal, healthy calves. With these promising results, somatic cell co-culture was soon adopted by many groups studying embryonic development of a wide variety of species. The benefits seen included faster cleavage, higher blastocyst rates, increased cell numbers, better hatching rates, increased resultant pregnancies, and most importantly, increased live births (reviewed in Orsi and Reischl 2007). However, co-culture was not a one-size fits all approach. A few drawbacks were quickly identified, including the differing media needs of cells and embryos, the possibility of viral transfection from cells to embryos, and the inherent struggles of reproducibility that come from using undefined biological substrates. One additional difficulty of co-culture is the often necessary inclusion of serum in the culture media, which has been previously established as a source of both positive and negative effects on the embryo.

A type of co-culture which has yet to show negative effects on embryo development is the common practice of group embryo culture. Although they are not somatic cells, they are still able to take from and contribute to the culture environment in a way that has over and over again been shown to be helpful to embryos. The origin of ruminant group culture may well be a holdover from in vitro murine embryo work (Hammond 1949), where multiple embryos develop together in vivo. As well, this tendency may have come as researchers realized that less development resulted from individual culture (Gardner *et al.* 1994). In any case, group culture often stands as the standard to which individual culture is compared.

Serum Added to Culture Media

Serum was found to have a beneficial effect on embryo development almost as soon as in vitro production methods were developed. At that time, due to the high level of interest in human IVF, and close ties between researchers in both human and animal fields, the additive of choice for researchers in some parts of the world (especially Australia) was human serum (Thompson *et al.* 2007). Researchers in other parts of the world used bovine serum. This provided an ample source of protein, growth factors, and other hormones. Owing to its biological origins, the exact composition of both human and ruminant source serum, however, differs from batch to batch and thus it remains an undefined medium. One study (Van Langendonck *et al.* 1997) compared embryos cultured in SOF with those cultured in SOF + 10% fetal bovine serum (FBS) showed that the development to blastocyst was faster and approximately doubled by the addition of serum (16% vs. 30% oocytes developed to blastocyst). This effect was maintained even when the SOF was supplemented with glucose (0.67 mM) (15% of oocytes developing to blastocysts) or the FBS was de-salted (components less than 5 kDa removed) (29% of oocytes developing to blastocysts) suggesting both that the increased development was due to components of serum which were over 5 kDa in mass, and not simply the added glucose. Gutierrez-Adan *et al.* (2001b) found a similar increase in development with SOF supplemented with serum (34.4% vs. 47.5% of cleaved embryos to blastocyst). This study also showed an interesting effect of serum on sex ratio. Whereas the blastocysts formed from embryos cultured in SOF tended to be around half (51%) males, those embryos cultured in SOF with serum had a statistically significant increase in males (57%). The same author showed in a different study that male embryos cultured in serum tend to develop faster, but the sex ratio is evened by day 10 in culture (Gutierrez-Adan *et al.* 2001a).

However, this increased development came at a cost, as investigators quickly saw a trend in heavier birth weights, termed large offspring syndrome (LOS). The human serum-cultured ovine offspring in a study by Walker *et al.* (1992) also showed other perturbations, such as longer gestation (which could not account for the increase in birth weight) and increased deaths in the perinatal period. These differences persisted in ruminant embryo culture even after the serum source was changed to fetal bovine serum. The mechanism of these changes is not fully understood, but is thought to be the result of impaired methylation of the embryonic genome. One gene which has been hypothesized to be involved in large offspring syndrome is insulin-like growth factor, an imprinted gene (Young *et al.* 2003). DNA methylation is an epigenetic change which is often involved in downregulating transcription. However, in sheep feti cultured in serum as embryos (and experiencing large offspring syndrome), a complete loss of methylation was found in the Insulin-like Growth Factor 2 receptor (IGF2R), and these same feti had a 30-60% *reduction* in IGF2R in tissues (Young *et al.* 2001). The researchers purported that the marked differences in body weight seen may be attributable to differences in IGF2 experienced during other times in gestation, or to differences in IGF2 binding protein concentrations. These changes are seen both in the preimplantation embryo (Fernandez-Gonzalez *et al.* 2004) as well as in the fetus (Young *et al.* 2001). Interesting work done in murine embryos cultured with either bovine serum or bovine serum albumin (BSA) suggests that pups who were cultured in serum as embryos continued to show differences up to nearly 2 years of age. The serum pups showed higher rates of pneumonia, larger organs (heart and liver), and differences in motor skills which persisted (Fernandez-Gonzalez *et al.* 2004). This study may have been confounded by the unusually poor development in the FBS compared to the BSA groups (43% vs 95%) as well as the lowered survival to parturition (23% vs. 44%). It is also important to note that this was a murine model; however, such long term studies have not been carried out in ruminants.

Soon after seeing these unfortunate outcomes of serum supplementation, investigators turned to other avenues which would lead to equivalent development without serum. One early study (Gardner *et al.* 1994) showed that healthy (measured by total cell numbers) blastocysts could be produced in serum-free conditions. However, they found several things necessary in the absence of serum: amino acids must be present in the media, the media must be changed frequently to lessen the concentration of ammonia, and the embryos must be cultured in groups. Additionally, many formulations of serum replacer are available today, which, while somewhat undefined (as the exact formulation is proprietary), should be a more consistent formula than FBS. Oocytes and embryos which were matured and cultured in serum substitute in human in vitro embryo production outperformed both in morphologic assessments and pregnancy rates their serum-containing counterparts (Staessen *et al.* 1990). Likewise, in the bovine research field of study, a comparison of oocytes matured in media containing serum replacer as well as epidermal growth factor, then cultured in SOF with serum replacer showed no differences in development to the blastocyst compared to the control group that was matured and cultured in media containing FBS (Sagirkaya *et al.* 2007). Another small study (n=6) showed no aberrant growth in calves born from embryos cultured in serum replacer (Moore *et al.* 2007).

Modern Media Choices

For all of the aforementioned reasons, modern embryo culture is trending toward cell-free, serum-free conditions. This results in more defined protocols which are potentially easier to replicate in different laboratories around the world. However, finding the right constituents of media which successfully support embryos through the 8 cell block and help them to form healthy blastocysts has proven a considerable task. Media additives for group culture which have shown some promise include defined amino acids (Gardner *et al.* 1994), transforming growth

factor β and basic fibroblast growth factor (Larson *et al.* 1992), and polyvinyl alcohol, insulin, transferrin, selenium, myo-inositol, and epidermal growth factor (Wang *et al.* 2012a).

Assessment of In Vitro Produced Embryo Quality and Health

There are several ways to objectively measure the health and quality of in vitro produced embryos. The most obvious measure is to transfer them to synchronized recipients and assess pregnancy and calving rate compared to in vivo produced embryos transferred to another group. Indeed, although this might serve as the “gold standard” test of embryo health, it is often not feasible or practical due to using oocytes of unknown genetic quality or financial constraints of research. Thus, less cumbersome methods of embryo quality assessment have necessarily arisen. There is a validated method of evaluating in vivo produced embryos (Stringfellow 2013) which was developed by members of the International Embryo Transfer Society. These guidelines concern percentage of cellular material which forms the embryonic cell mass as well as the stage of development compared to the expected stage. They serve to inform decisions about transferring, freezing, and discarding in vivo produced embryos.

The IETS grading system allows assessment of live embryos intended for transfer. However, since many research projects have blastocyst development (rather than pregnancy establishment) as their endpoint, researchers can use more invasive methods of measuring embryo health which are not concerned with embryo survival. Jiang *et al.* (1992) applied cell counting techniques to in vitro produced bovine embryos for the first time (having been previously applied to the pig by Papaioannou and Ebert (1988)). The results validated this method as they showed that higher morphologically graded embryos had higher total cell counts. The cell counting method was advantageous because it removed the subjective element of embryo grading. As well, embryo grading has been shown to be a poorer predictor of pregnancy

establishment in in vitro (vs in vivo) produced embryos (Sugimura *et al.* 2012). At around the same time, Iwasaki *et al.* (1990) applied the immunosurgery techniques to the bovine embryo which had been developed previously in mouse embryos (Handyside and Hunter 1984). This technique involved selectively permeabilizing the nuclei of the trophoctoderm layer of the embryo to allow fluorescent stains to penetrate, which should leave the inner cell mass (ICM) nuclei intact and only penetrable by vital stains. As a result, differences between in vivo and in vitro cultured embryos were illuminated, as in vivo embryos had higher total cell as well as ICM numbers than their in vitro cultured counterparts in each developmental stage examined. Van Soom *et al.* (1997) compared timing of blastocyst development to total and ICM cell number, and found that later developing blastocysts tended to have fewer total ICM cells, a trend which was true of both in vitro and in vivo produced embryos. As this method became more popular and more data was gathered, in vivo produced embryos consistently showed that 30-40% of their total nuclei were made up by inner cell mass cells, and this number became a benchmark by which in vitro produced embryos were measured.

Individual Embryo Culture

Most of the early work to optimize bovine embryo culture systems treated these embryos in a group fashion. There was little necessity, it seemed, to culture them individually. However, the idea of following an individual oocyte all the way through embryo development is only possible with a robust individual embryo production system.

That individually cultured ruminant embryos are hampered in development compared to their group cultured counterparts is a fact discovered shortly after in vitro systems were developed (Gardner *et al.* 1994). This was not an unexpected result as a similar phenomenon had already been reported in murine embryo culture (Wiley *et al.* 1986). The most popular

hypothesis to explain this phenomenon is that embryos are secreting autocrine and paracrine factors during development, and these factors are diluted by the culture media itself when culturing small numbers of embryos. In a variety of species, these factors include insulin like growth factor I, transforming growth factor α (Paria and Dey 1990) and β (Lim and Hansel 1996), epidermal growth factor (Paria and Dey 1990), platelet-activating factor (O'Neill 1997), and platelet derived growth factor (Thibodeaux *et al.* 1995; Lim and Hansel 1996). The logical test of the dilution theory would be very small volume embryo culture. In a two-part experiment, Ferry *et al.* (1994) first maintained embryo:culture volume ratios and found no difference in development between 10 embryos cultured in 10 μL and 40 embryos cultured in 40 μL . However, when a similar embryo:volume ratio was used for single embryo culture (1 embryo: 5 μL), the embryos failed to develop to the blastocyst stage. Not surprisingly, failure was seen at the ratios of 1:10 and 1:40 as well. Carolan *et al.* (1996) found a similar result with individual embryos cultured in 1 μL of media showing complete failure to develop. Culture media volume is a bit of a double-edged sword, as in addition to diluting the aforementioned embryotropic factors, culture media also dilutes embryotoxic factors such as ammonia and reactive oxygen species. Out of these unpromising beginnings, researchers searched for ways to improve the development of embryos cultured individually.

Media Additives for Single Embryo Culture

One of the most active areas in single embryo culture research is in the media. Indeed, this is an active area of research for embryo culture in general, and as our understanding of media for group culture has improved, individual embryo culture has reaped some benefits. In creating more and more complete types of media, the individually cultured embryo finds itself in less and less need of fellow embryos. Serum was a natural first choice in media additive (despite the

drawbacks discussed earlier), as perhaps the negative effects of serum would not be so detrimental to individual embryos. One experiment (Carolan *et al.* 1996) pitted individually cultured embryos against their group-cultured counterparts (both culture media contained serum). The results showed no statistical difference between the development to blastocyst of group (n=20-25) and individually cultured embryos. However, a significant difference was found in the hatching rate of individual embryos cultured in 20 μ L droplets vs. group cultured embryos (no difference was found in hatching rates of individuals cultured in 10 μ L droplets vs. group). Another investigation (Mizushima and Fukui 2001) compared group cultured embryos with serum to individual embryos in serum, as well as individual embryos in polyvinyl alcohol (PVA) (as a substitute for albumin) alone, with hypotaurine, β -mercaptoethanol, or both and found that the development rate of individual and group embryos cultured in 10% serum was not significantly different, but both were significantly higher than any of the PVA medias. A serum-free media was developed by Wydooghe *et al.* (2013) in which supplementation with insulin, transferrin, and selenium raised development to the blastocyst to the level of group culture (35% vs. 31% zygote to blastocyst). However, the hatching rate was significantly depressed when compared to group cultured embryos with serum (12.5% vs. 34% blastocysts to hatched blastocysts).

Other investigations into individual embryo culture media have focused on the use of somatic cell co-culture (despite the concerns mentioned previously). An interesting study by Goovaerts *et al.* (2011) compared blastocyst development of individually cultured embryos with a co-culture of either heterologous or autologous cumulus cells to group cultured embryos. The autologous culture removes one major problem usually inherent in co-culture – namely, the possibility of viral transfection from somatic cell to embryo. In both cases, the individually cultured oocytes showed significantly less development than the group comparison. However, in

a related experiment (Goovaerts *et al.* 2009), the same group showed that culturing embryos individually either over a previously established monolayer of cumulus cells or adding cumulus cells to culture droplets at the same time as the embryo resulted in development to blastocyst rates which were not significantly different than group cultured embryos (38.2% on monolayer, 31.9% with addition of cumulus cells with embryo, 36.4% group). These studies present promising results which suggest that co-culture may be able to create an environment similar to that provided by group culture. One limitation of both of the aforementioned studies is that they used freshly collected granulosa cells for culture, which presents a problem for any lab without high throughput.

Physical Changes to the Culture Environment

As media for culture more closely approximated that found *in vivo*, other researchers continued to refine the physical culture environment to reflect the *in vivo* conditions as well. In the oviduct, the embryo is surrounded by a very small amount of fluid and in a relative microenvironment when compared to the vast space of the uterus. Thus, two schemes of emulating the *in vivo* environment were developed: microfluidics and microwells.

Microfluidics is the name given to creating an environment for an embryo in which the media surrounding the embryo can be changed without moving the embryo itself. It is an intricate collaboration between scientists and engineers. Earlier work showed that although moving embryos from one media to another lowered the amount of ammonia and other toxins in the media, they had less eventual development, purportedly due to the sudden shock of media transition (Fukui *et al.* 1996). Microfluidics aims to lessen the shock of media transition by making media changes very slowly and allowing the embryo to adjust. Further, these frequent gradual media changes allow the embryo to rest in very small volumes without the negative

effects of increased concentrations of byproducts and wastes (Krisher and Wheeler 2010). Furthermore, media changes can be made without physical manipulation of the embryo, which may reduce stress (Wheeler *et al.* 2007). The system setup requires the input and expertise of engineers, but the results of these systems have so far been promising in both bovine (Krisher and Wheeler 2010) and other species (Glasgow *et al.* 2001; Beebe *et al.* 2002).

Another type of physical change to the culture environment is the microwell. The first report of microwells for aggregation of embryonic cells was published in the early nineties (Wood *et al.* 1993). Wood advocated the “darning needle” aggregation technique as a simpler way of creating chimaeras by incubating embryonic stem cells with zona-free morulas in a microwell. The advantages of this system, as compared to blastocyst injection, were that the skills required and specialized equipment needed were far less. Indeed, it only required a blunted needle to make small depressions in the bottom of culture dishes to keep the non-zona enclosed cells aggregated. A few years later, the very similar Well of the Well (WOW) system was described by Vajta *et al.* (2000). The WOW system was originally intended as a system for zona-free embryo growth and development but researchers quickly saw its value in zona-intact embryos as well. The WOW system is created by making a small depression in the bottom of a culture system (either multi-well plates or dishes with droplets) which creates a small microenvironment for the embryo that is still part of a much larger macroenvironment (Figure 1). This idea was very attractive for the culture of zona-free embryos as it provides an environment for keeping the cells aggregated in the absence of a zona pellucida whose function, among others, is maintaining cell-cell contact. The first article published presented impressive results, with single embryos having a blastocyst/zygote rate of 60% compared to a group culture rate of 55% (Vajta *et al.* 2000). The conclusion of the study was that the microwell provided an environment which would allow the paracrine factors secreted by embryos to remain closer to the embryo,

which may mimic the concentrations of these factors found in group cultures, while also being part of a larger macroenvironment which allows the diffusion of waste products and reactive oxygen species. Another study showed that the WOW system may increase embryo competency, as the recipient pregnancy rates at both 30 and 60 days were significantly higher in WOW-produced embryos than control in vitro produced embryos (Sugimura *et al.* 2010).

Microwells as a production system for in vitro embryo culture has been replicated by different investigators in laboratories all over the world (Akagi *et al.* 2010; Hoelker *et al.* 2010; Dai *et al.* 2012; Sugimura *et al.* 2013). In addition to comparing culture droplets to microwell culture, the original work (Vajta *et al.* 2000) compared the culture of single and multiple microwells per droplet. It is well established that in standard culture conditions, embryos cultured singly versus those cultured in groups show vast differences in development (i.e. Gardner *et al.* 1994). The results from the research performed by Vajta *et al.* (2000) showed no statistical difference in embryo development in single and multiple microwells per droplet. The blastocyst rate demonstrated by the microwell system was similar to that demonstrated in the group culture. This suggests that the benefits gained from the microwell system are not enhanced or diminished by the presence or absence of more embryos. From the perspective of single embryo culture, this is a promising development. However, this lack of difference between single and multiple microwells has not been a consistent finding. For instance, in a study involving in vivo derived murine embryos, Dai *et al.* (2012) found similar results between embryos cultured in groups and those in a multiple microwell system. Further, there was comparable development between embryos cultured singly and in a single microwell system. However, the group systems showed significantly improved development over both single culture systems by 10-15%.

Importance of In Vitro Production to the cattle industry

According to the latest IETS statistics (2013), 1,877,400 oocytes were collected from 128,444 donors largely obtained from ultrasound-guided ovum pickup with a much smaller proportion received from the abattoir. This resulted in 457,455 embryos. Over 350,000 of these embryos originated from South America, mostly Brazil. The total number of embryos collected worldwide in 2012 was 1,143,119. The fact that in vitro produced embryos now account for nearly 1 of every 2 embryos produced worldwide should fuel investigators everywhere to develop improved methods of embryo production which are suitable both for the lab as well as for use in clinical practice.

The established difficulties in individual embryo culture have been partially or fully overcome in a variety of ways which were discussed previously. Continuing in that vein, the current investigation intends to build on previous research to find if any of several treatments is successful in improving individual embryo culture to equal the development rate seen in group culture. The treatments of interest included the addition of serum, serum replacer, or co-culture to the culture media, as well as the inclusion of microwells in the culture environment.

Chapter 2: Manuscript

Abstract

Individually cultured bovine embryos have shown a reduced ability to proceed through development to the blastocyst stage when compared to cultures containing multiple embryos. The origin of this deficit is not entirely clear, although it is suspected to be due to a dilution of autocrine/paracrine factors secreted by the zygotes and embryos themselves. Individual embryo culture is sometimes necessary, as in the case of low oocyte recovery, or when individual embryo identification is needed. Thus, the aim of this project was to evaluate the effects on individual bovine embryo development of adding granulosa cells and/or two types of serum to the culture media. Our hypothesis was that addition of granulosa cells, 10% fetal bovine serum, knockout SR serum replacer, or a combination of serum and cells would improve development to the blastocyst stage of individually cultured bovine embryos up to the level of development seen in group culture. Ovaries were obtained from a local abattoir and all visible follicles aspirated. The recovered oocytes were washed, evaluated and selected for integrity of cumulus cell layers, and individually matured in maturation media for 23 ± 1 h. Oocytes were then moved to individual fertilization droplets and 25,000 frozen-thawed motile sperm were added to each droplet and co-cultured for 18 h. Then, the presumptive zygotes were randomly divided into 6 treatment groups with a 3x2 design. A culture of frozen-thawed mitomycin-c treated bovine granulosa cells was established in the bottom of droplets in half of the treatment groups. Both groups, with and without granulosa cells, were further divided into three serum treatment groups. The embryo culture media (Synthetic oviduct fluid) was altered by the addition of 10% fetal bovine serum, KnockOut™ serum replacer, or not modified. Embryos were assessed on day 8 post fertilization to determine blastocyst development, and on days 10-11 post fertilization to evaluate hatching success. A separate group of oocytes was matured, fertilized, and cultured in a group (n=10) as a

standard with which to compare the treatment groups. The results showed that none of the blastocyst rates of the six treatment groups were statistically different from each other (avg. 4.7%), with the group culture being significantly higher than all of them (21.7%) (logistic regression). When aggregated, none of the treatments had a significant effect on blastocyst rate in individual culture. However, a significant difference was found in the hatching rate between blastocysts cultured in media including cells (71.4%) and those cultured without cells (18.2%). This suggests that although the addition of granulosa cells did not increase the number of blastocysts formed, it increased the health and viability of the ones which did form. A second experiment was conducted in much the same manner, but instead of culture media treatments the physical structure of the culture environment was changed to the well-of-the-well (WOW) system. This time the oocytes were matured and fertilized in groups, then divided into one of four groups arranged in a 2x2 design: single or multiple (n=10) embryos and presence or absence of WOW depressions. No difference was found in development to the blastocyst in any of the 4 groups. The hatching rate of blastocysts cultured in the group WOW system was equivalent to those in the group condition, significantly higher than those in the individual droplet group, and trending ($p=0.07$) toward higher than those in the individual WOW condition. The differences found in hatching rate suggest that using the WOW system as an individually identifiable culture system in vitro may be a viable alternative to individual culture.

Introduction

From the earliest reported attempts to culture embryos in vitro (Hammond 1949), 40 years passed before the development of the first calf from a completely in vitro process (Eyestone and First 1989). In the 25 years since that seminal development, the research in this area has experienced incredible growth in the number of publications. Thus an average of 76 articles were published annually between 1985-89, which rose to an average of 186 in the most recent 5 year

period (PubMed search for “bovine in vitro embryo”, July 16, 2014). Soon after in vitro embryo production became possible, researchers realized that ruminant embryos developed better in the company of other embryos, and that embryos produced individually experienced depressions in development (Gardner *et al.* 1994). One study showed no difference in eventual development between oocytes matured individually vs. in groups, then grouped for fertilization and culture (Carolan *et al.* 1996). Further data from the same study suggested that in larger fertilization droplet volume, no deleterious effect on development was found with oocytes fertilized individually. This leaves the culture step as the probable limitation of further development for individual embryos. Although the exact cause of this depression in development has yet to be elucidated, most researchers attribute it to autocrine/paracrine factors which are severely diluted in single embryo culture. If this hypothesis could explain this aspect of embryo culture, it would follow that keeping the embryo to culture media ratio constant should in effect solve the problem faced by single embryos. However, studies have repeatedly shown that single embryos need more than just a low volume culture drop to match the development found in group culture (Ferry *et al.* 1994; Carolan *et al.* 1996). Although a murine study showed equivalent development, key markers of embryo health were different suggesting that something more than culture volume must be varied in order to attain equivalent development in single embryo culture (Vutyavanich *et al.* 2011). Another angle of research concerns conditioned media – perhaps culturing individual embryos in media which surrounded other embryos or cells (thus potentially containing an undefined amount of these autocrine/paracrine factors) would support growth better than fresh media. Although this has shown some promise with groups of embryos (Eyestone and First 1989), it has not been as successful in single embryo work (Goovaerts *et al.* 2009). Despite these known difficulties in development, single embryo culture is sometimes necessary due to low recovery from procedures such as ovum pick-up or postmortem oocyte recovery. Further, it may

be advantageous from a research point of view to follow single oocytes all the way through development.

If a robust single embryo culture system is not possible with current techniques, perhaps embryos could be cultured in an individually identifiable manner while still conserving the benefits of group culture. A system was described by Vajta *et al.* (2000) which involves creating small depressions in the bottom of culture environments. This well-of-the-well (WOW) system is purported to create a microenvironment in which the concentration of autocrine/paracrine factors can increase while still remaining part of the larger macroenvironment for the purpose of waste dilution. Despite the lack of evidence to substantiate the hypothesis, this method has proven itself in individually identifiable embryo production in labs all around the world (Akagi *et al.* 2010; Hoelker *et al.* 2010; Dai *et al.* 2012; Sugimura *et al.* 2013).

This study aimed to improve on existing methods of individual in vitro embryo production. The first experiment examined the addition of both serum, serum replacer, and frozen-thawed mitomycin-c treated granulosa cells to culture medium to try to improve the development rate of embryos cultured singly. The goal of the second experiment was to test a method called well-of-the-well and the possibility of group culturing embryos in an individually identifiable manner. The hypothesis of experiment 1 was that the inclusion of serum, serum replacer, or granulosa cell co-culture in the culture media would increase development of individually matured, fertilized, and cultured embryos to the level seen with group maturation, fertilization, and culture. The hypothesis of experiment 2 was that embryos cultured in the well-of-the-well system either in groups or individually would show increased development to the level seen in group culture.

Materials and Methods

Experiment 1

Preparation of the culture environment

A culture of granulosa cells which had been removed from a previous group of bovine oocytes was established and passaged twice in tissue culture. They were treated with mitomycin-c to prevent further replication and then frozen in liquid nitrogen for future use. On day -2 (day 0 = fertilization), 1×10^6 of these cells were thawed and resuspended in 5 mL of cell culture media (Table 1). Hoechst staining previously showed these cells retained 88% of their viability after thawing. The mixture was centrifuged at 150g for 5 minutes, then the supernatant was removed and the pellet was resuspended in 1.1 mL cell culture media (Table A-1) to give a concentration of 4.5×10^5 cells per mL, which resulted in approximately 100% confluence at the bottom of 30 μ L droplets. The cell mixture was used in half of the individual culture dishes, whereas in the other half (as well as the group culture dish) cell culture media without cells was used. In all cases, 30 μ L droplets of media were established in 60 mm tissue culture dishes under embryo grade mineral oil (Sigma). These dishes were incubated at 38.5°C in humidified air with 5% CO₂. On day -1, the cell culture media was removed from all droplets and replaced with 30 μ L phosphate buffered saline (PBS) as a wash step. Then the PBS was removed and replaced with 30 μ L of one of 3 media. One of 3 additives were included in the culture media (Table A-2): 10% FBS, 10% KnockOut™ serum replacer (Gibco) or no additive. The different media were allocated to droplets in equal numbers, creating a 2x3 experimental design with presence or absence of granulosa cells varying within the different culture media (Table 1). The droplets for the group cultured zygotes were washed in the same manner and culture medium with no additive was added as the final medium. All of the culture dishes were returned to the incubator until presumptive zygote addition on day 1.

In vitro embryo production

Ovaries were obtained from a local abattoir and transported back to the laboratory and in warmed saline (25-35 °C) and processed within 7 h of slaughter. All visible follicles 2-15 mm in size were aspirated with an 18 GA needle connected to a 12 cc syringe. The follicular fluid and cumulus-oocyte complexes (COC) were pooled in 50 mL conical vials and allowed to settle for 10 minutes. The pellet which formed on the bottom of the vial was aspirated and resuspended in 30-40 mL of warmed (38.5 °C) SOF-HEPES (Table A-3), and again allowed to settle for 10 minutes. The pellet was aspirated and placed in to a 100 mm diameter search dish with an equal volume of SOF-HEPES. COCs which were surrounded by at least 2 complete layers of cumulus cells were selected and washed in 3 successive dishes of SOF-HEPES. No further selection took place during the experiment. The selected COC were then placed either individually in 10 µL droplets under mineral oil or grouped in the same maturation media (Table A-4) in a 35 mm dish and incubated at 38.5°C in humidified air with 5% CO₂ for 22-24 hours. After maturation, the oocytes were washed 3 times in SOF-HEPES and then placed either individually into droplets of fertilization media (Table A-5) which were 24 µL or placed in groups of 10 in droplets of 48 µL. These droplets were hemispheric in shape, made by placing half the volume of media in the petri dish, covering them with mineral oil, then injecting the remaining volume. Frozen-thawed semen from the same bull was used throughout the experiment. Two straws of semen were thawed and layered on top of a discontinuous Percoll gradient (45%/90%), then centrifuged at 700 g for 15 minutes. The overlying media was removed from the pellet and the pellet was resuspended in 5 mL SOF-HEPES, then again centrifuged at 150g for 10 minutes. The resultant pellet was resuspended in SOF-HEPES to attain a final motile sperm concentration of 2.5×10^7 /mL. Then 1 µL of the sperm solution was added to each individual fertilization droplet and 2 µL was added to each group droplet. The oocytes and sperm were coincubated at 38.5°C in humidified air with 5% CO₂ for approximately 18 hours. After coincubation, the presumptive zygotes were washed

in SOF-HEPES and then vortexed in SOF-HEPES for 3 minutes to remove all cumulus cells. The presumptive zygotes were then washed 3 additional times in SOF-HEPES, after which they were placed individually in 30 μ L droplets in one of six individual treatment conditions described earlier or in groups of 10 in 30 μ L droplets. The cultures were maintained for 7 days at 38.5°C in a sealed modular incubator which was filled with a gas mixture of 5% O₂, 5% CO₂, 90% N₂. Embryo development was assessed on day 8 (day 0 = fertilization) for development to the blastocyst stage. After this evaluation, half of the day 8 blastocysts were removed from culture for cell differential determination. The remaining blastocysts were returned to the incubator (maintained at 38.5°C in humidified air with 5% CO₂) to be assessed on day 10-11 for hatching.

Differential staining procedure

This cell differential protocol was developed at the University of Florida (Ozawa *et al.* 2011). Amounts of all reagents were approximately 200 μ L unless otherwise specified, and all incubations took place at room temperature unless otherwise specified. The protocol was carried out in 500 μ L Eppendorf tubes in which the embryos stayed as the reagents were added and removed. On day 8 embryos were removed from culture and fixed in 4% paraformaldehyde for 15 minutes. Then the embryos were washed with a cold solution of PBS/Polyvinyl pyrrolidone (PVP) (0.2g PVP added to 100 mL PBS) 3 times. After washing, the embryos were incubated with a permeabilization solution of 0.25% Triton-X-100 in PBS for 20 minutes. Then, they were again washed, this time with a solution of 0.1 mL Tween-20 and 0.1g BSA added to 100 mL PBS (hereafter called “wash buffer”). The washed embryos were incubated in a blocking buffer made of 5g BSA added to 100 mL PBS for 1 hour. Afterward, the embryos were incubated in 20 μ L of an anti-CDX2 antibody (Biogenex) overnight at 4C. The next day they were washed 3 times in the wash buffer and then incubated with 20 μ L of FITC labeled anti-mouse IgG for 1 hour in the dark. The embryos were again washed 3 times in wash buffer and then placed on glass slides

where residual media was removed and replaced with 15 μL of a DAPI/anti fade solution (Invitrogen). A glass coverslip was applied and sealed with clear nail polish. Then slides were examined for fluorescence using an Olympus CKX41 microscope fitted with a Lumenera Infinity 3 camera. DAPI staining should equally stain all nuclei within the embryo, and this was evaluated by visible excitation with a blue emission when 400-418 nm light was used on the specimen. FITC stained nuclei, which should only represent the trophectoderm cells (due to the CDX-2 antibody labeling followed by anti-mouse FITC labeled IgG), were excited by 478-495 nm light and emitted a green hue. Thus by counting nuclei visible in each image, it was possible to determine total cell number as well as trophectoderm cell number, and from that infer inner cell mass (ICM) cell number and the proportion of total cells which comprise the ICM (ICM percentage).

Experiment 2

Preparation of well-of-the-well culture dishes

The well-of-the-well (WOW) instrument used in this experiment was a stainless steel instrument originally meant for small animal dentistry. The original tip was removed and ground to a point in which the diameter was approximately 100 μm . The tip of the point was blunted and the entire end of the instrument polished to remove any microscopic roughened areas. The instrument was autoclaved before each use. Culture media in 30 μL droplets were established under oil in the bottom of 60 mm tissue culture dishes. In half of the dishes, depressions were created with the WOW instrument by using steady manual pressure along with lateral (not rotational) movement of the handle to bring the diameter opening to approximately 300 μm with an apparently similar depth (Figure 1). This method of WOW making resulted in very little debris left in the droplet itself and created a rather consistent depression. In $\frac{1}{4}$ of the dishes, 1

depression was made per droplet, and in another ¼, 10 depressions were made per droplet. In half of the droplets, no depressions were made.

In vitro embryo production

The basic structure of experiment 2 is very similar to the first experiment. Briefly, COCs (n=180 per group, total n=720) were isolated and selected in an identical manner, and after washing, were matured in groups of 200-300 in 2.5 mL maturation media. After 22-24 hours, these COCs were washed and moved in groups of 10 into 50 µL droplets of fertilization media. Each droplet had 50,000 sperm added and oocytes were coincubated with sperm for 18 hours. At the conclusion of the coincubation period, the presumptive zygotes were washed and then exposed to a vortex to denude them of cumulus cells. Then the presumptive zygotes were divided into one of four culture conditions: well-of-the-well or conventional droplets and individual or multiple (n=10) culture resulting in a 2x2 experimental design (Table 2). The embryos were checked for development to the blastocyst stage on day 8 (day 0 = day of fertilization) and for hatching on day 10.

Statistical analysis

Statistical analysis of development was carried out using PROC Logistic (SAS Institute, Inc., Cary, NC, version 9.3). Cell number and differential data was analyzed with PROC Glimmix. Significance was considered when $p < 0.05$. Contrasts were used for all between group comparisons, and p-values were transformed using the Benjamin-Hochberg adjustment.

Results

Experiment 1

A total of 5 replicates were completed. However, one of them (replicate 4) resulted in significantly lower development across all treatments as well as the group control, and was

therefore removed from further statistical analysis. The remaining 4 replicates resulted in development in the group condition which was not significantly different between replicates. The total number of oocytes included in the study was 812 over 4 replicates. Of these, 120 were in the group condition and 692 were in one of the 6 individual treatments (Table 3). A logistical regression model examining the development rate between treatments and the group condition revealed that embryos in the group condition experienced significantly higher rates of development when compared to all of the treatment conditions, and none of the treatments were significantly different from each other (Figure 2). When aggregated by presence or absence of granulosa cells (regardless of additive), the group condition remained significantly higher than both treatment conditions, and the presence or absence of granulosa cells did not show a significant difference in development (Figure 3). Likewise, when aggregated by media additive (regardless of presence or absence of cells), the group condition was significantly different than all of the experimental treatments, and the treatments were not significantly different from each other (Figure 4).

Two measures of embryo health (hatching rate and ICM percentage) were also explored. Considering the embryos that were left in culture to assess hatching (n=30), hatching rate of all of the treatment groups individually revealed no differences between all treatments and the group condition (Figure 5). However, when aggregated by presence or absence of granulosa cells, those embryos which developed in the presence of cells were as likely as those in the group condition, and significantly more likely than those cultured without cells to hatch (Figure 6). The ICM percentage of the fixed blastocysts (n=24) was likewise analyzed by treatment, but no differences were found between treatments or the group condition (Figure 7). When pooled by presence or absence of granulosa cells, a numerical pattern is shown similar to the hatching rate, with cell

presence and the group condition equivalent with absence of granulosa cells smaller – however, this difference was found to be nonsignificant ($p=0.3$) (Figure 8).

Experiment 2

A total of 6 replicates were completed comparing traditional group embryo culture and 2 variations of the WOW culture system. Replicates 1-3 experienced bacterial contamination, difficulty in making the depressions, and extremely low development in all groups and thus were removed from further statistical analysis. After many attempts to improve development which included changing nearly all of the stock solutions, water source, method of CO₂ acclimation, and method of making depressions, development in replicates 4-6 improved somewhat, but only to about half the level of that seen in the group condition of experiment 1. Thus, although similar trends are seen between the two experiments (realizing that the group condition and treatment 6 in experiment 1 are equivalent to groups A and B, respectively, in experiment 2), the actual numbers are quite different. In experiment 1 development in the group condition outpaced treatment 6 by a rate of approximately 4 to 1. In experiment 2, the development difference in those same 2 conditions (this time called A and B) was reduced to 2 to 1. The blastocyst rate by treatment is displayed in Figure 9, and none of the differences between groups was found to be significant. However, the hatching rate data ($n=32$) shown in Figure 10 showed that blastocysts from group C (group WOW) were much more likely to hatch than those from group B (individual droplet) as well as a trend toward an increased likelihood when compared to group D (individual WOW) ($p=0.07$).

Discussion

The present study aimed to improve on methods of individual embryo culture. Although a single treatment which would raise the development of individual embryos to the level of group

counterparts was not found, this investigation stands to contribute to the overall body of knowledge and help future researchers zero in on the key elements to consistently high development in single embryo culture. It is clear from this as well as similar investigations (i.e. Wiley *et al.* 1986; Ferry *et al.* 1994; Gardner *et al.* 1994; Carolan *et al.* 1996; Goovaerts *et al.* 2010) that group culture still holds an advantage over individual culture. However, even group culture with defined media is somewhat undefined because of the unknown level of production of factors from the other embryos. As well, in the case of a droplet containing a degenerating or dead embryo which is hypothesized to spread its waste products to all of the other embryos in the droplet, the potential disadvantages of group culture are easy to see. However, with current capabilities the possible negative aspects of group culture are more than made up for in the very real disadvantages of individual culture.

The development rate of oocytes subjected to IVM, IVF, and IVC to the blastocyst is often used to compare relative success of different treatments within as well as sometimes between investigations. The first study to successfully produce a fully in vitro blastocyst (Eyestone and First 1989) reported a very respectable 17.3% as the morula/blastocyst per total oocyte rate in the best treatment. Since that time, the definition of adequate or acceptable development to blastocyst has risen slightly, but most researchers consider success if rates greater than 20-25% are achieved, and development often plateaus around 40% (Mermillod *et al.* 2006). Some investigators report a rate of blastocysts per cleaved embryo as a way of eliminating the incompetent or unfertilized oocytes from the analysis. This causes difficulty when attempting to compare the results of different studies. The system of embryo production employed in the current study relied on SOF-based media and resulted in a total group development to the blastocyst rate of 21.7 +/- 3.76%. Other investigations (Lim *et al.* 2007) have reported rates somewhat higher than that reported here. Those investigators reported development rates of 52%

using defined, sequential media; the results reported in the current study are still comparable to those rates. Thus, this display of adequate development suggests that the much lower development displayed in all of the treatment groups reflects not a failure of the overall embryo production system employed, but a deficiency of individual embryo culture systems.

Low individual embryo development

Considering reasons for the reduced development in the individual treatment groups brings a number of possibilities to mind. First, there was a numerical trend of serum or serum replacer in co-culture to have a negative effect on development. Groups 1, 2, and 3 all contained granulosa cells in the embryo culture droplets; the difference between them lay in their additive, which was serum, serum replacer, and nothing, respectively. Development in these three groups was 2.56, 2.61, and 7.76%, respectively. Second, groups 2 and 5 both had serum replacer added to the media, but group 2 also had granulosa cells, whereas 5 did not. Development in those groups was 2.61 and 7.02%, respectively. Although these changes were not statistically significant, they point to possible antagonistic relationships between serum (or replacer) and culture with granulosa cells. However, no such antagonistic relationship was found in research conducted by Goovaerts *et al.* (2012).

The development of individually cultured embryos with various media additives compared to their group counterparts was lower than expected. These results suggest that fellow oocytes are providing a factor (and more likely several factors) which either serum, serum replacer, or co-culture is not providing. Despite the negative aspects associated with adding serum to embryo culture media, serum is undeniably an ample source of albumin, growth factors, and hormones. Which factors are present and in what proportions is less clear; given the batch to batch variability (Honn *et al.* 1975), the resistance associated with serum use is justified. Indeed, the results of this study fail to replicate the improvement in development shown in previous

studies (Van Langendonck *et al.* 1997; Gutierrez-Adan *et al.* 2001b). It is prudent to note that these particular investigations studied group-cultured embryos. Thus a logical conclusion could be that the beneficial effects of serum are mediated by fellow embryos and that serum cannot exert its beneficial effects on embryos cultured individually. Another possibility is that, given the previously mentioned batch to batch variability, the serum used in this project may have been deficient in some necessary growth factor. While this is possible, it seems unlikely as the serum used was replaced each month of the project and the serum-supplemented groups maintained consistently low levels of development among replicates. Additionally, serum replacer is a manufactured, defined medium whose components should not change from batch to batch. Thus similar results would be expected in repeated experiments using different batches of serum replacer. Another possible reason is that the addition of serum or serum replacer in this study was made before the presumptive zygotes were added to the culture droplets, however, other studies have found benefits in delaying the serum addition until 2 (21 vs 12% day 6 blastocysts per oocyte in serum added after the start of culture vs. without) (Carolan *et al.* 1995) or 5 (40 vs 22% blastocyst per oocyte in serum added after the start of culture vs. without) (Thompson *et al.* 1998) days post fertilization.

The results of this study show that single embryo culture is far from an easy process. In spite of a relatively robust group embryo production system (demonstrated in experiment 1), the development of single embryos remained poor despite several different treatments. In contrast to the results of previous work (Goovaerts *et al.* 2009; Goovaerts *et al.* 2011), a co-culture of bovine cumulus cells was not successful in improving development of individually cultured embryos. This discrepancy invites a number of possible reasons why – among them, previous work used a fresh culture of granulosa cells (rather than frozen-thawed) as well as normally dividing cells (rather than mitomycin-c treated). The modifications were made in this experiment for practical

reasons, such as not having a ready source of fresh granulosa cells (as the culture plates were made the day before ovary arrival). Further, preventing normal cell division by pre-freeze treatment of the cells with mitomycin c was carried out to prevent abundant cellular replication which could deplete the nutrients of the media more quickly than non-replicating cells. However, it is entirely possible that some of the benefit of somatic cell co-culture is exhibited by the somatic cells dividing and growing normally. Further, mitomycin c may change the transcriptome of treated cells (Wang *et al.* 2012b), meaning that treated cells may make different products than untreated cells. This has not been tested in bovine granulosa cells, however its possible contribution to the conflicting results cannot be ignored. Although they showed an excellent rate of survival post-freeze (88%), no investigation was undertaken to explore the potential differences between these cells and granulosa cells freshly removed from a follicle. Indeed, other experiments have shown that the steroidogenic production of bovine granulosa cells can be manipulated in culture by simply altering the plating density (Portela *et al.* 2010). Characterizing what the granulosa cells are adding to or taking away from the media represents a needed area of future research.

Embryos which were cultured in the presence of granulosa cells were significantly more likely to hatch than their counterparts cultured without granulosa cells. Although the results show no increase in development with addition of cumulus cells, the blastocysts which are formed appear to be healthier. Hatching rate may be a bit of an artificial measure, as different mechanisms may be required in vitro than are used in vivo (i.e. in murine, Montag *et al.* 2000). However, it remains clear that viability as well as continued growth is a prerequisite for hatching.

The fact that in vitro embryo production as a whole is moving toward more defined media and away from things like serum and culture with granulosa cells has been discussed previously. Thus, this investigation may seem in some ways like a step backward. However, the

preponderance of the evidence suggests that group and single embryo culture are two very different things. In an effort to improve individual culture techniques, each pathway, even those which have already been traversed in the pursuit of group culture, must be explored. Although this investigation did not reveal a better method, it does raise interesting questions about why the addition of serum appears to benefit group-cultured embryos but may not provide the same benefit to individual embryos.

The results of experiment 2 in comparison to experiment 1 were not as expected. A once robust group culture system which was exactly the same as that used in the first experiment experienced a profound depression in development. The reasons for this change are not entirely clear. Avenues explored to try to increase development include water source, replacement of all organic stock solutions, replacement of amino acid solutions, and new ways of media CO₂ acclimation. Other research groups at the university who received ovaries from the same source during the same period reported very good development, suggesting that the problem did not lie in the abattoir or transportation to the lab. The 5% O₂ 5% CO₂ 90% N₂ tank was the same as that used in experiment 1 with good development, suggesting that the gaseous environment was not the problem. All of the oocyte and embryonic manipulations were carried out by the author using the same protocol each time. Further, both experiments were conducted between November and February, when follicular quality should not be negatively affected by heat stress (reviewed in Wolfenson *et al.* 2000; Ferreira *et al.* 2011). Thus, the reason behind the poor development remains a mystery. Although expected trends are still present, it is not possible to fully evaluate the efficacy of the WOW system in this laboratory because larger or smaller differences may have been masked by the low development rate. Despite this low development, a very interesting trend emerged in the hatching rate data, where despite statistically similar development data in all 4 conditions, the group cultured (droplet or WOW) embryos showed a

significantly higher likelihood to hatch. Thus, although conclusions are hard to draw from the development data, the hatching rate data suggests that embryos cultured in the absence of other embryos are hindered in further development in a way which the WOW system does not make up for. The WOW system in effect provides the ability to follow development of individual embryos while those same embryos are receiving the benefit of group culture. Indeed, one research group has fabricated a ready-made WOW plate (Akagi *et al.* 2010), when and if it becomes commercially available, it may increase the interest in this system as it removes some of the variability and challenges that come with preparing the WOW depressions themselves.

Chapter 3: Conclusion

Further research in this field should focus on developing culture systems which reliably result in developed embryos, no matter how many are in the group. Defined media is the direction of current research in conventional group culture, and may indeed be the right direction for individual embryo production as well. Defined media take away some of the variability inherent in embryo production systems. It is easy to imagine a time when individual embryo culture will be the method of choice given the high level of media control given to the practitioner. The group WOW system in this study showed itself to be a good alternative to individual embryo production in the form of individually identifiable embryo culture. Thus, although the WOW system may provide an individually identifiable transition, the future of individual embryo culture likely lies in culturing them one by one.

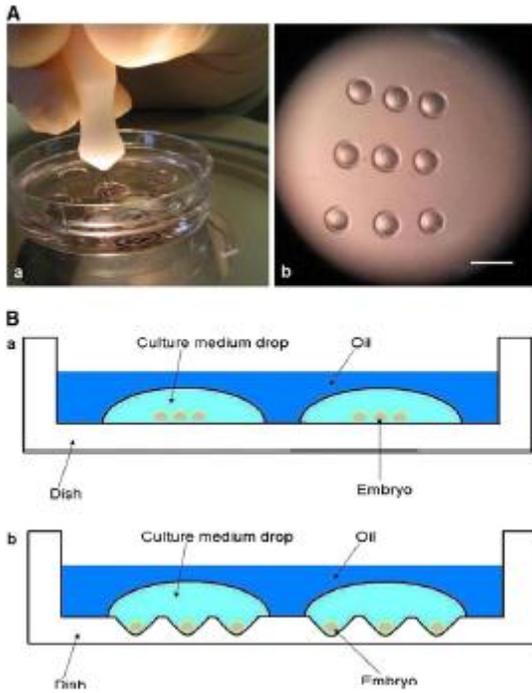


Figure 1. Illustration of the Well-of-the-Well System compared to the traditional culture drop system.

From Dai *et al.* (2012) (Used with permission)

A: a: Creating the depressions; b: microscopic appearance of the microwells

B: a: traditional droplet culture system; b: microwell or Well-of-the-Well system

Table 1. Individual Culture Treatment Conditions in Experiment 1.

	IVC + 10% FBS	IVC + 10% SR	IVC
Granulosa cells	1	2	3
No cells	4	5	6

IVC: Culture medium (Table B) FBS: Fetal Bovine Serum SR: Serum Replacer

Table 2. Treatment Conditions in Experiment 2.

	Multiple zygotes (n=10)	Single zygote
Conventional droplet	A	B
Well-of-the-Well droplet	C	D

Conventional droplet: 30 μ L culture media under oil, Well-of-the-Well droplet: 30 μ L culture media under oil with a single or multiple (n = 10) microwells measuring approximately 300 μ m diameter.

Table 3. Number of oocytes per group in Experiment 1: Comparison of additives (FBS, SR or no additive) in a culture system with or without granulosa cell co-culture.

	IVC + 10% FBS	IVC + 10% SR	IVC
Granulosa cells	117	115	116
No cells	115	114	115

IVC: Culture medium (Table A-2) FBS: Fetal Bovine Serum SR: Serum Replacer

Total of 4 replicates. n = 692 oocytes in treatment groups, with an additional 120 in the group control

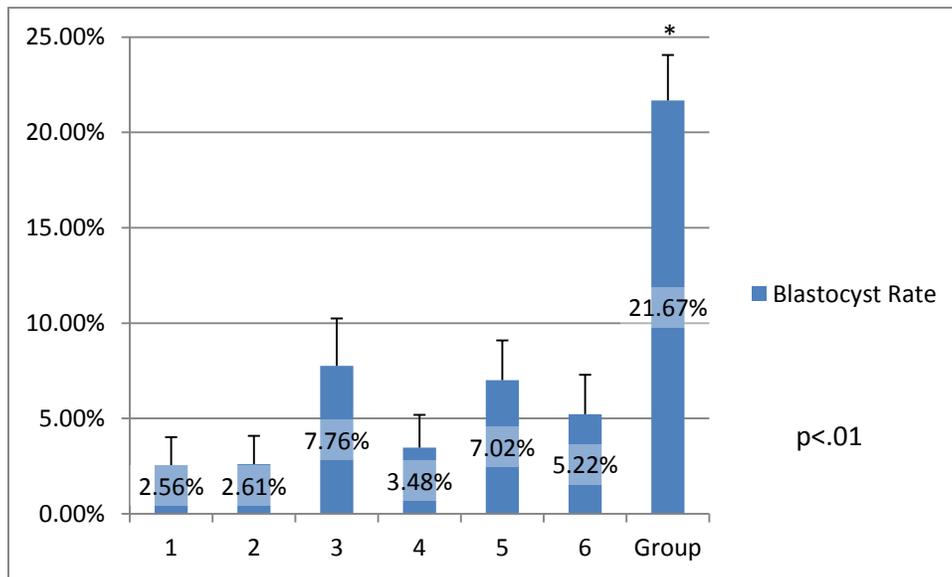


Figure 2. Experiment 1: Rate of Development to Blastocyst per Oocyte by Treatment.

Data analyzed via logistic regression in comparison to the group condition, and treatments were compared to each other by contrasts. Results showed that the group condition was significantly different than all treatment conditions, but no other significant differences were found between treatments. Treatments: (all numbered treatments involved embryos cultured individually) 1 = serum additive plus co-culture; 2 = serum replacer additive plus co-culture; 3 = no additive plus co-culture; 4 = serum additive without co-culture; 5 = serum replacer additive without co-culture; 6 = no additive or co-culture; Group = 10 oocytes cultured together without additive or co-culture.

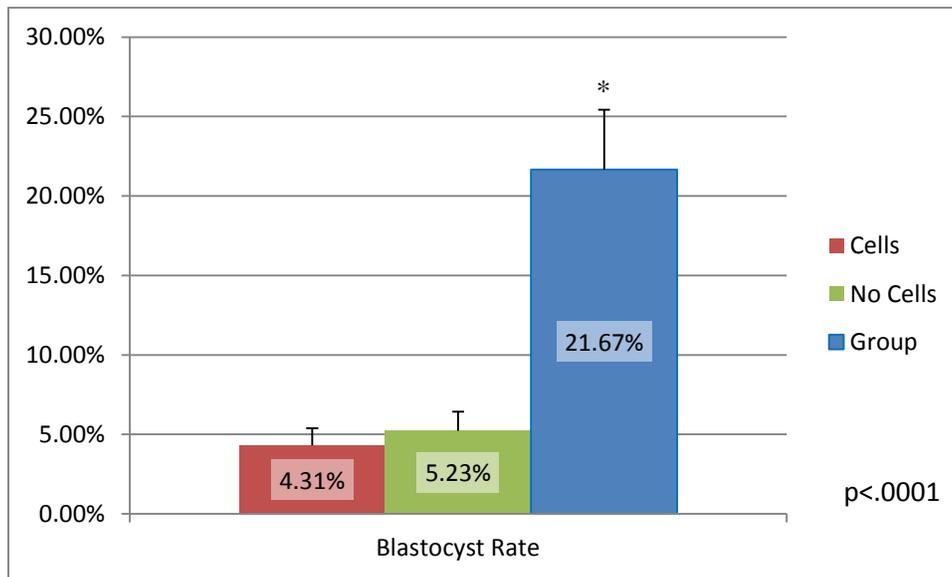


Figure 3. Experiment 1: Development Rate Pooled by Presence/Absence of Granulosa Cells Independent of Media Additive.

Data analyzed via logistic regression in comparison to group condition, and contrasts were used to compare treatments to each other. Group cultured embryos had significantly greater development than those cultured in either experimental condition, and the presence or absence of granulosa cells was not found to cause a significant difference in development. Cells = individual embryos cultured in the presence of a co-culture of granulosa cells; No Cells = individual embryos cultured in the absence of a co-culture of granulosa cells; Group = 10 oocytes cultured together without additive or co-culture.

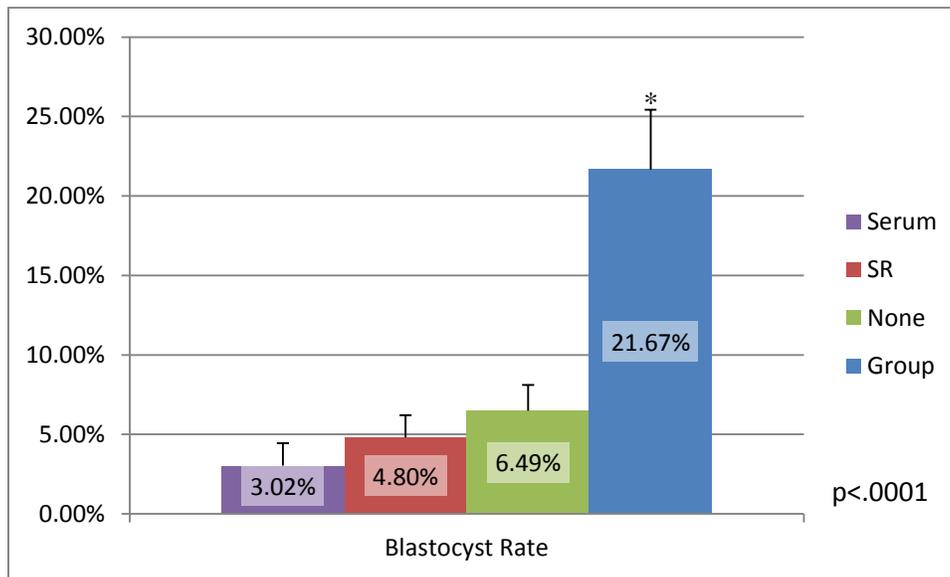


Figure 4. Experiment 1: Development Rate Pooled by Media Additive Independent of Presence or Absence of Granulosa Cell Co-Culture.

Analyzed via logistic regression in comparison to group condition, and contrasts were used to compare treatment groups to each other. Significantly higher development was found in the group condition than all treatments, and no significant differences were found comparing the treatment groups to each other. Serum = 10% fetal bovine serum added to culture media; SR = 10% KnockOut™ serum replacer added to culture media; None = no additive to culture media; Group = 10 oocytes cultured together without additive or co-culture.

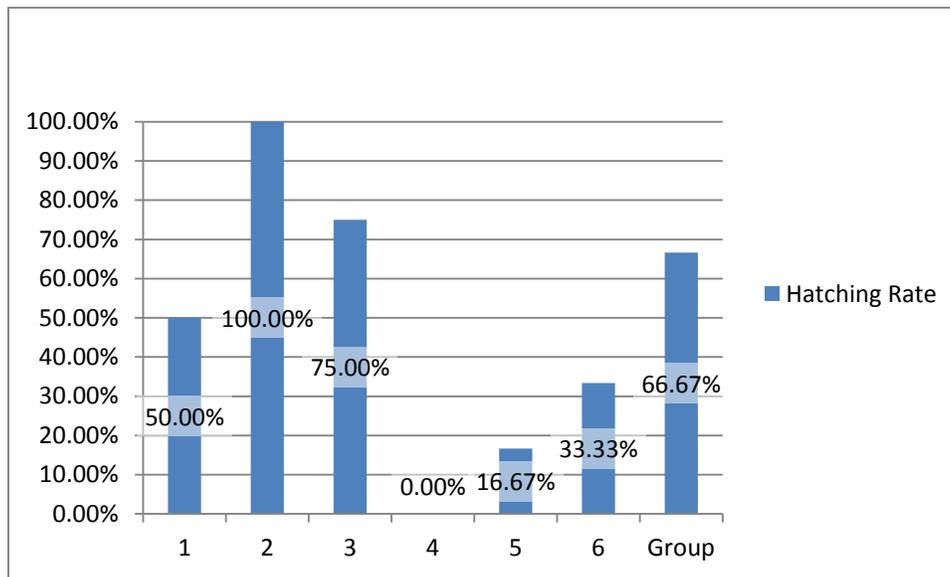


Figure 5. Experiment 1: Hatching Rate by Treatment.

Analyzed by logistic regression in comparison to the group condition, and contrasts were used to compare treatments to each other. No significant difference was found between any treatment or group condition. Treatments: (all numbered treatments involved embryos cultured individually) 1 = serum additive plus co-culture; 2 = serum replacer additive plus co-culture; 3 = no additive plus co-culture; 4 = serum additive without co-culture; 5 = serum replacer additive without co-culture; 6 = no additive or co-culture; Group = 10 oocytes cultured together without additive or co-culture.

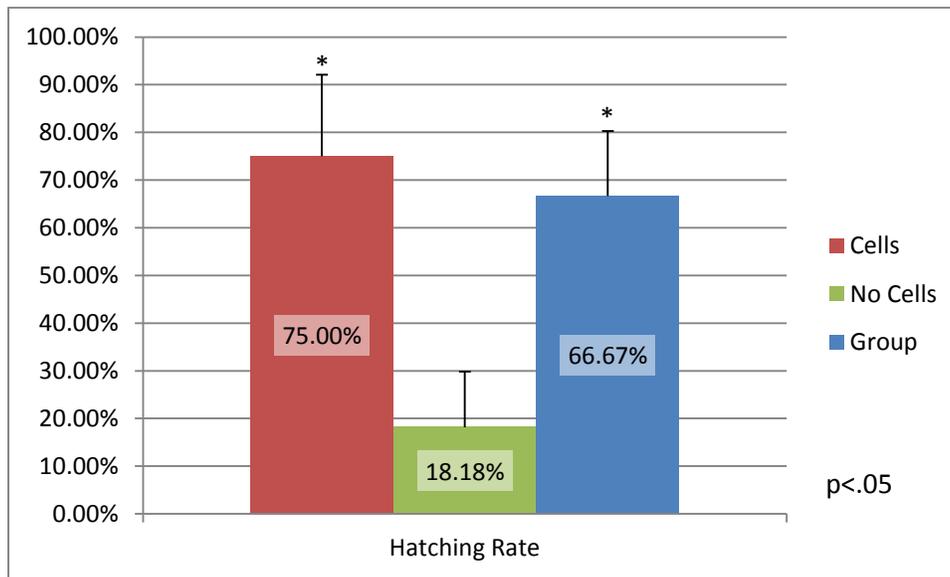


Figure 6. Experiment 1: Hatching Rate Pooled by Presence/Absence of Granulosa Cells Independent of Media Additive.

A significant difference was found between both presence of cells and group condition vs. absence of cells. The hatching rate of those embryos cultured individually with granulosa cells was statistically equivalent to that of the group condition. Cells = individual embryos cultured in the presence of a co-culture of granulosa cells; No Cells = individual embryos cultured in the absence of a co-culture of granulosa cells; Group = 10 oocytes cultured together without additive or co-culture.

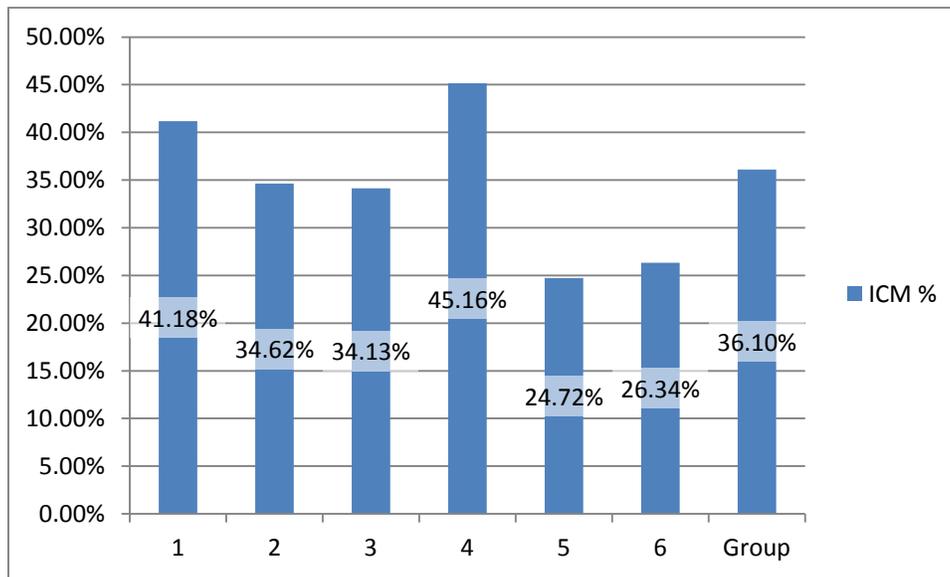


Figure 7. Experiment 1: Inner Cell Mass Percentage by Treatment.

Data analyzed by one way ANOVA. No significant differences were found between any treatment or the group condition. Treatments: (all numbered treatments involved embryos cultured individually) 1 = serum additive plus co-culture; 2 = serum replacer additive plus co-culture; 3 = no additive plus co-culture; 4 = serum additive without co-culture; 5 = serum replacer additive without co-culture; 6 = no additive or co-culture; Group = 10 oocytes cultured together without additive or co-culture.

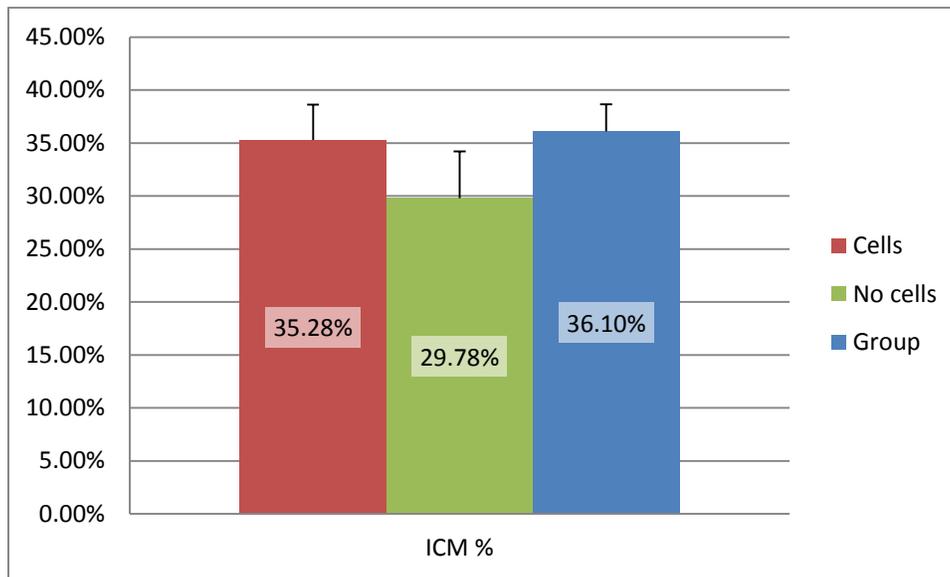


Figure 8. Experiment 1: Inner Cell Mass Percentage Pooled by Presence/Absence of Cells Independent of Media Additive.

Data analyzed by one-way ANOVA. Comparison of presence vs. absence of cells was not significant ($p=0.24$). ICM % = Percentage of the total number of cells in the blastocyst which make up the inner cell mass; Cells = individual embryos cultured in the presence of a co-culture of granulosa cells; No Cells = individual embryos cultured in the absence of a co-culture of granulosa cells; Group = 10 oocytes cultured together without additive or co-culture.

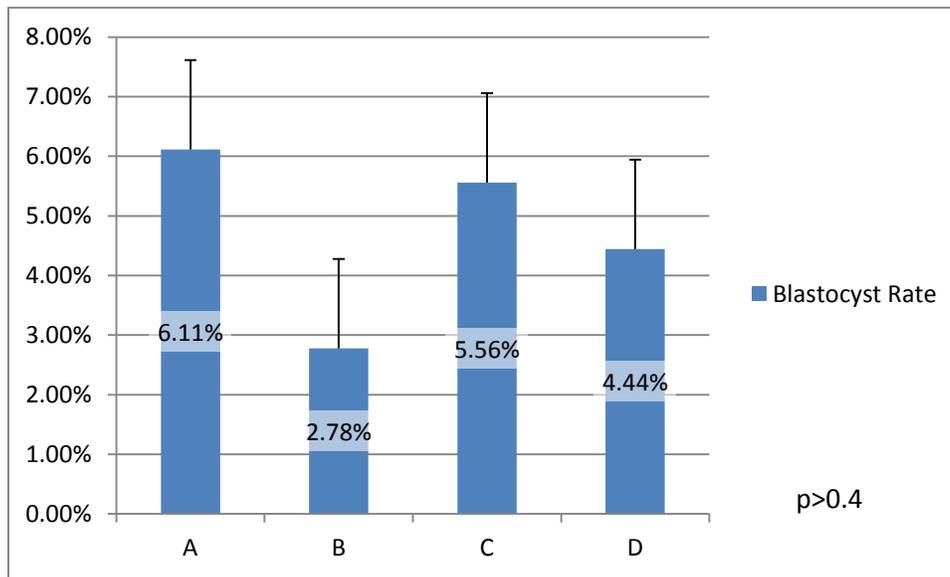


Figure 9. Experiment 2: Development Rate to Blastocyst per Oocyte by Treatment.

Data analyzed by logistical regression in comparison to the group condition (Treatment A) with contrasts to determine differences between treatments. No significant differences were found. Treatments: A = 10 embryos cultured together in a 30 μ L droplet; B = 1 embryo cultured individually in a 30 μ L droplet; C = 10 embryos cultured in a 30 μ L droplet with 10 microwells; D = single embryo cultured in a 30 μ L droplet with a single microwell.

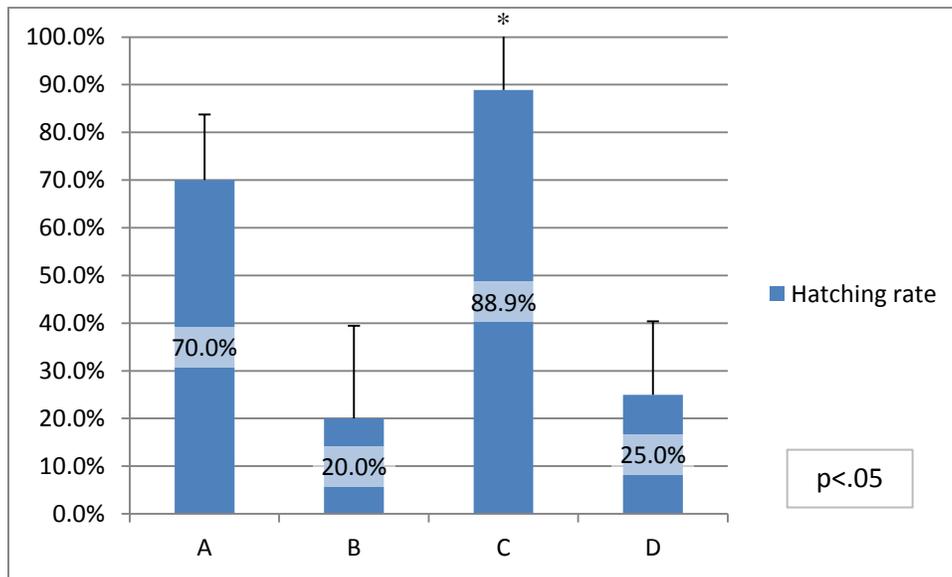


Figure 10. Experiment 2: Hatching Rate by Treatment.

Total number of blastocysts evaluated = 32. Data analyzed by logistic regression in comparison to the group condition (Treatment A) with contrasts to determine differences between groups. A significant difference was found between treatments B and C. Treatments: A = 10 embryos cultured together in a 30 μ L droplet; B = 1 embryo cultured individually in a 30 μ L droplet; C = 10 embryos cultured in a 30 μ L droplet with 10 microwells; D = single embryo cultured in a 30 μ L droplet with a single microwell.

References

- Akagi, S., Hosoe, M., Matsukawa, K., Ichikawa, A., Tanikawa, T., and Takahashi, S. (2010) Culture of bovine embryos on a polydimethylsiloxane (PDMS) microwell plate. *J Reprod Dev* **56**(4), 475-9
- Beebe, D., Wheeler, M., Zeringue, H., Walters, E., and Raty, S. (2002) Microfluidic technology for assisted reproduction. *Theriogenology* **57**(1), 125-35
- Biggers, J.D., Gwatkin, R.B., and Brinster, R.L. (1962) Development of mouse embryos in organ cultures of fallopian tubes on a chemically defined medium. *Nature* **194**, 747-9
- Brackett, B.G., Bousquet, D., Boice, M.L., Donawick, W.J., Evans, J.F., and Dressel, M.A. (1982) Normal development following in vitro fertilization in the cow. *Biol Reprod* **27**(1), 147-58
- Brackett, B.G., Hall, J.L., and Oh, Y.K. (1978) In vitro fertilizing ability of testicular, epididymal, and ejaculated rabbit spermatozoa. *Fertil Steril* **29**(5), 571-82
- Brock, H., and Rowson, L.E. (1952) The production of viable bovine ova. *The Journal of Agricultural Science* **42**(04), 479-481
- Caixeta, E.S., Machado, M.F., Ripamonte, P., Price, C., and Buratini, J. (2013) Effects of FSH on the expression of receptors for oocyte-secreted factors and members of the EGF-like family during in vitro maturation in cattle. *Reprod Fertil Dev* **25**(6), 890-9
- Camous, S., Heyman, Y., Meziou, W., and Menezo, Y. (1984) Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. *J Reprod Fertil* **72**(2), 479-85
- Carolan, C., Lonergan, P., Khatir, H., and Mermillod, P. (1996) In vitro production of bovine embryos using individual oocytes. *Mol Reprod Dev* **45**(2), 145-50
- Carolan, C., Lonergan, P., Van Langendonck, A., and Mermillod, P. (1995) Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization in vitro. *Theriogenology* **43**(6), 1115-28
- Chang, M.C. (1951) Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature* **168**(4277), 697-8
- Chang, M.C. (1959) Fertilization of rabbit ova in vitro. *Nature* **184**(Suppl 7), 466-7

Clemente, M., de La Fuente, J., Fair, T., Al Naib, A., Gutierrez-Adan, A., Roche, J.F., Rizos, D., and Lonergan, P. (2009) Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction* **138**(3), 507-17

Dai, S.J., Xu, C.L., Wang, J., Sun, Y.P., and Chian, R.C. (2012) Effect of culture medium volume and embryo density on early mouse embryonic development: tracking the development of the individual embryo. *J Assist Reprod Genet* **29**(7), 617-23

Dieleman, S.J., Hendriksen, P.J., Viuff, D., Thomsen, P.D., Hyttel, P., Knijn, H.M., Wrenzycki, C., Kruip, T.A., Niemann, H., Gadella, B.M., Bevers, M.M., and Vos, P.L. (2002) Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre-implantation embryos. *Theriogenology* **57**(1), 5-20

Dowling, D.F. (1949) Problems of the transplantation of fertilized ova. *The Journal of Agricultural Science* **39**(04), 374-396

Downs, S.M., and Eppig, J.J. (1984) Cyclic adenosine monophosphate and ovarian follicular fluid act synergistically to inhibit mouse oocyte maturation. *Endocrinology* **114**(2), 418-27

Edwards, R.G. (1965) Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature* **208**(5008), 349-51

Eppig, J.J. (1989) The participation of cyclic adenosine monophosphate (cAMP) in the regulation of meiotic maturation of oocytes in the laboratory mouse. *J Reprod Fertil Suppl* **38**, 3-8

Eyestone, W.H., and First, N.L. (1989) Co-culture of early cattle embryos to the blastocyst stage with oviducal tissue or in conditioned medium. *J Reprod Fertil* **85**(2), 715-20

Eyestone, W.H., and First, N.L. (1991) Characterization of developmental arrest in early bovine embryos cultured in vitro. *Theriogenology* **35**(3), 613-24

Fernandez-Gonzalez, R., Moreira, P., Bilbao, A., Jimenez, A., Perez-Crespo, M., Ramirez, M.A., Rodriguez De Fonseca, F., Pintado, B., and Gutierrez-Adan, A. (2004) Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. *Proc Natl Acad Sci U S A* **101**(16), 5880-5

Ferreira, R.M., Ayres, H., Chiaratti, M.R., Ferraz, M.L., Araujo, A.B., Rodrigues, C.A., Watanabe, Y.F., Vireque, A.A., Joaquim, D.C., Smith, L.C., Meirelles, F.V., and Baruselli, P.S. (2011) The low fertility of repeat-breeder cows during summer heat stress is related to a low oocyte competence to develop into blastocysts. *J Dairy Sci* **94**(5), 2383-92

Ferry, L., Mermillod, P., Massip, A., and Dessy, F. (1994) Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. *Theriogenology* **42**(3), 445-53

Fukui, Y., Lee, E.S., and Araki, N. (1996) Effect of medium renewal during culture in two different culture systems on development to blastocysts from in vitro produced early bovine embryos. *J Anim Sci* **74**(11), 2752-8

Gandolfi, F., and Moor, R.M. (1987) Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. *J Reprod Fertil* **81**(1), 23-8

Gardner, D.K., Lane, M., Spitzer, A., and Batt, P.A. (1994) Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod* **50**(2), 390-400

Glasgow, I.K., Zeringue, H.C., Beebe, D.J., Choi, S.J., Lyman, J.T., Chan, N.G., and Wheeler, M.B. (2001) Handling individual mammalian embryos using microfluidics. *IEEE Trans Biomed Eng* **48**(5), 570-8

Goovaerts, I.G., Leroy, J.L., Jorssen, E.P., and Bols, P.E. (2010) Noninvasive bovine oocyte quality assessment: possibilities of a single oocyte culture. *Theriogenology* **74**(9), 1509-20

Goovaerts, I.G., Leroy, J.L., Langbeen, A., Jorssen, E.P., Bosmans, E., and Bols, P.E. (2012) Unravelling the needs of singly in vitro-produced bovine embryos: from cumulus cell co-culture to semi-defined, oil-free culture conditions. *Reprod Fertil Dev* **24**(8), 1084-92

Goovaerts, I.G., Leroy, J.L., Rizos, D., Bermejo-Alvarez, P., Gutierrez-Adan, A., Jorssen, E.P., and Bols, P.E. (2011) Single in vitro bovine embryo production: coculture with autologous cumulus cells, developmental competence, embryo quality and gene expression profiles. *Theriogenology* **76**(7), 1293-303

Goovaerts, I.G., Leroy, J.L., Van Soom, A., De Clercq, J.B., Andries, S., and Bols, P.E. (2009) Effect of cumulus cell coculture and oxygen tension on the in vitro developmental competence of bovine zygotes cultured singly. *Theriogenology* **71**(5), 729-38

Gutierrez-Adan, A., Granados, J., Pintado, B., and De La Fuente, J. (2001a) Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured in vitro. *Reprod Fertil Dev* **13**(5-6), 361-5

Gutierrez-Adan, A., Lonergan, P., Rizos, D., Ward, F.A., Boland, M.P., Pintado, B., and de la Fuente, J. (2001b) Effect of the in vitro culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. *Theriogenology* **55**(5), 1117-26

Hammond, J., Jr. (1949) Recovery and culture of tubal mouse ova. *Nature* **163**(4131), 28

Handyside, A.H., and Hunter, S. (1984) A rapid procedure for visualising the inner cell mass and trophoctoderm nuclei of mouse blastocysts in situ using polynucleotide-specific fluorochromes. *J Exp Zool* **231**(3), 429-34

Hashimoto, S., Minami, N., Yamada, M., and Imai, H. (2000) Excessive concentration of glucose during in vitro maturation impairs the developmental competence of bovine oocytes after in vitro fertilization: relevance to intracellular reactive oxygen species and glutathione contents. *Mol Reprod Dev* **56**(4), 520-6

Heyman, Y., Camous, S., Fevre, J., Meziou, W., and Martal, J. (1984) Maintenance of the corpus luteum after uterine transfer of trophoblastic vesicles to cyclic cows and ewes. *J Reprod Fertil* **70**(2), 533-40

Hoelker, M., Rings, F., Lund, Q., Phatsara, C., Schellander, K., and Tesfaye, D. (2010) Effect of embryo density on in vitro developmental characteristics of bovine preimplantative embryos with respect to micro and macroenvironments. *Reprod Domest Anim* **45**(5), e138-45

Holt, W.V., Hernandez, M., Warrell, L., and Satake, N. (2010) The long and the short of sperm selection in vitro and in vivo: swim-up techniques select for the longer and faster swimming mammalian sperm. *J Evol Biol* **23**(3), 598-608

Honn, K.V., Singley, J.A., and Chavin, W. (1975) Fetal bovine serum: a multivariate standard. *Proc Soc Exp Biol Med* **149**(2), 344-7

Hunter, R.H., Lawson, R.A., and Rowson, L.A. (1972) Maturation, transplantation and fertilization of ovarian oocytes in cattle. *J Reprod Fertil* **30**(2), 325-8

IETS (2013) 2012 Statistics of Embryo Collection and Transfer in Domestic Farm Animals. International Embryo Transfer Society.

Iwasaki, S., Yoshiba, N., Ushijima, H., Watanabe, S., and Nakahara, T. (1990) Morphology and proportion of inner cell mass of bovine blastocysts fertilized in vitro and in vivo. *J Reprod Fertil* **90**(1), 279-84

Jiang, H.S.W.W.L.L.K.H.G.I.P.C. (1992) Examination of cell numbers of blastocysts derived from IVM, IVF and IVC of bovine follicular oocytes. *Theriogenology* **37**(1), 229

Kepkova, K.V., Vodicka, P., Toralova, T., Lopatarova, M., Cech, S., Dolezel, R., Havlicek, V., Besenfelder, U., Kuzmany, A., Sirard, M.A., Laurincik, J., and Kanka, J. (2011) Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition. *Theriogenology* **75**(9), 1582-95

Krisher, R.L., and Wheeler, M.B. (2010) Towards the use of microfluidics for individual embryo culture. *Reprod Fertil Dev* **22**(1), 32-9

Larson, R.C., Ignatz, G.G., and Currie, W.B. (1992) Transforming growth factor beta and basic fibroblast growth factor synergistically promote early bovine embryo development during the fourth cell cycle. *Mol Reprod Dev* **33**(4), 432-5

Leibfried-Rutledge, M.L., Critser, E.S., Eyestone, W.H., Northey, D.L., and First, N.L. (1987) Development potential of bovine oocytes matured in vitro or in vivo. *Biol Reprod* **36**(2), 376-83

Lim, J.M., and Hansel, W. (1996) Roles of growth factors in the development of bovine embryos fertilized in vitro and cultured singly in a defined medium. *Reprod Fertil Dev* **8**(8), 1199-205

Lim, K.T., Jang, G., Ko, K.H., Lee, W.W., Park, H.J., Kim, J.J., Lee, S.H., Hwang, W.S., Lee, B.C., and Kang, S.K. (2007) Improved in vitro bovine embryo development and increased efficiency in producing viable calves using defined media. *Theriogenology* **67**(2), 293-302

Lonergan, P., Fair, T., Corcoran, D., and Evans, A.C. (2006) Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. *Theriogenology* **65**(1), 137-52

Lonergan, P., Rizos, D., Gutierrez-Adan, A., Fair, T., and Boland, M.P. (2003) Effect of culture environment on embryo quality and gene expression - experience from animal studies. *Reprod Biomed Online* **7**(6), 657-63

Mermillod, P., Locatelli, Y., Dalbiès-Tran, R., Uzbekova, S., Baril, G., Guignot, F., Perreau, C., Poulin, N., Touzé, J., and Pennetier, S. In vitro production of ruminant embryos: results, limits and perspectives. 2006,

Mizushima, S., and Fukui, Y. (2001) Fertilizability and developmental capacity of bovine oocytes cultured individually in a chemically defined maturation medium. *Theriogenology* **55**(7), 1431-45

Montag, M., Koll, B., Holmes, P., and van der, V. (2000) Significance of the number of embryonic cells and the state of the zona pellucida for hatching of mouse blastocysts in vitro versus in vivo. *Biol Reprod* **62**(6), 1738-44

Moore, K., Rodriguez-Sallaberry, C.J., Kramer, J.M., Johnson, S., Wroclawska, E., Goicoa, S., and Niasari-Naslaji, A. (2007) In vitro production of bovine embryos in medium supplemented with a serum replacer: effects on blastocyst development, cryotolerance and survival to term. *Theriogenology* **68**(9), 1316-25

Noda, Y., Matsumoto, H., Umaoka, Y., Tatsumi, K., Kishi, J., and Mori, T. (1991) Involvement of superoxide radicals in the mouse two-cell block. *Mol Reprod Dev* **28**(4), 356-60

O'Neill, C. (1997) Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro. *Biol Reprod* **56**(1), 229-37

Orsi, N.M., and Reischl, J.B. (2007) Mammalian embryo co-culture: trials and tribulations of a misunderstood method. *Theriogenology* **67**(3), 441-58

Ozawa, M., Carambula, S., Ealy, A., Sakatani, M., and Hansen, P. (2011) Immunolocalization of CDX2 in Bovine Embryos - A Method for Differential Counting of Trophectoderm and Inner Cell Mass Cells. In '.)

Papaioannou, V.E., and Ebert, K.M. (1988) The preimplantation pig embryo: cell number and allocation to trophectoderm and inner cell mass of the blastocyst in vivo and in vitro. *Development* **102**(4), 793-803

Paria, B.C., and Dey, S.K. (1990) Preimplantation Embryo Development Invitro - Cooperative Interactions among Embryos and Role of Growth-Factors. *Proc Natl Acad Sci U S A* **87**(12), 4756-4760

Parrish, J.J. (2014) Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. *Theriogenology* **81**(1), 67-73

Parrish, J.J., Susko-Parrish, J.L., and First, N.L. (1985) Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm in vitro. *Theriogenology* **24**(5), 537-49

Parrish, J.J., Susko-Parrish, J.L., and Graham, J.K. (1999) In vitro capacitation of bovine spermatozoa: role of intracellular calcium. *Theriogenology* **51**(2), 461-72

Pincus, G. Observations on the development of cow ova in vivo and in vitro. In 'Proc. First Nat. Egg-transfer Breed Conf', 1951, p. 18

Portela, V.M., Zamberlam, G., and Price, C.A. (2010) Cell plating density alters the ratio of estrogenic to progestagenic enzyme gene expression in cultured granulosa cells. *Fertil Steril* **93**(6), 2050-5

Restall, B.J., and Wales, R.G. (1966) The fallopian tube of the sheep. 3. The chemical composition of the fluid from the fallopian tube. *Aust J Biol Sci* **19**(4), 687-98

Rodriguez-Martinez, H. (2007) Role of the oviduct in sperm capacitation. *Theriogenology* **68**, Supplement 1(0), S138-S146

Rodriguez, K.F., and Farin, C.E. (2004) Gene transcription and regulation of oocyte maturation. *Reprod Fertil Dev* **16**(1-2), 55-67

Rose-Hellekant, T.A., Libersky-Williamson, E.A., and Bavister, B.D. (1998) Energy substrates and amino acids provided during in vitro maturation of bovine oocytes alter acquisition of developmental competence. *Zygote* **6**(4), 285-94

Sagirkaya, H., Misirlioglu, M., Kaya, A., First, N.L., Parrish, J.J., and Memili, E. (2007) Developmental potential of bovine oocytes cultured in different maturation and culture conditions. *Anim Reprod Sci* **101**(3-4), 225-40

Scaramuzzi, R.J., Baird, D.T., Campbell, B.K., Driancourt, M.A., Dupont, J., Fortune, J.E., Gilchrist, R.B., Martin, G.B., McNatty, K.P., McNeilly, A.S., Monget, P., Monniaux, D., Vinoles, C., and Webb, R. (2011) Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reprod Fertil Dev* **23**(3), 444-67

Schoenfelder, M., Schams, D., and Einspanier, R. (2003) Steroidogenesis during in vitro maturation of bovine cumulus oocyte complexes and possible effects of tri-butyltin on granulosa cells. *J Steroid Biochem Mol Biol* **84**(2-3), 291-300

Senger, P.L. (2005) 'Pathways to pregnancy and parturition.' 2nd rev. edn. (Current Conceptions: Pullman, WA) xii, 373 p.

Sirard, M.A., Parrish, J.J., Ware, C.B., Leibfried-Rutledge, M.L., and First, N.L. (1988) The culture of bovine oocytes to obtain developmentally competent embryos. *Biol Reprod* **39**(3), 546-52

Staessen, C., Van den Abbeel, E., Carle, M., Khan, I., Devroey, P., and Van Steirteghem, A.C. (1990) Comparison between human serum and Albuminar-20 (TM) supplement for in-vitro fertilization. *Hum Reprod* **5**(3), 336-41

Stringfellow, D.A.G.M.D.I.E.T.S. (2013) 'Manual of the International Embryo Transfer Society : a procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures.'

Sugimura, S., Akai, T., Hashiyada, Y., Aikawa, Y., Ohtake, M., Matsuda, H., Kobayashi, S., Kobayashi, E., Konishi, K., and Imai, K. (2013) Effect of embryo density on in vitro development and gene expression in bovine in vitro-fertilized embryos cultured in a microwell system. *J Reprod Dev* **59**(2), 115-22

Sugimura, S., Akai, T., Hashiyada, Y., Somfai, T., Inaba, Y., Hirayama, M., Yamanouchi, T., Matsuda, H., Kobayashi, S., Aikawa, Y., Ohtake, M., Kobayashi, E., Konishi, K., and Imai, K. (2012) Promising system for selecting healthy in vitro-fertilized embryos in cattle. *PLoS One* **7**(5), e36627

Sugimura, S., Akai, T., Somfai, T., Hirayama, M., Aikawa, Y., Ohtake, M., Hattori, H., Kobayashi, S., Hashiyada, Y., Konishi, K., and Imai, K. (2010) Time-lapse cinematography-compatible polystyrene-based microwell culture system: a novel tool for tracking the development of individual bovine embryos. *Biol Reprod* **83**(6), 970-8

Telford, N.A., Watson, A.J., and Schultz, G.A. (1990) Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev* **26**(1), 90-100

Tervit, H.R., Whittingham, D.G., and Rowson, L.E. (1972) Successful culture in vitro of sheep and cattle ova. *J Reprod Fertil* **30**(3), 493-7

Tesfaye, D., Lonergan, P., Hoelker, M., Rings, F., Nganvongpanit, K., Havlicek, V., Besenfelder, U., Jennen, D., Tholen, E., and Schellander, K. (2007) Suppression of connexin 43 and E-cadherin transcripts in in vitro derived bovine embryos following culture in vitro or in vivo in the homologous bovine oviduct. *Mol Reprod Dev* **74**(8), 978-988

Thibodeaux, J.K., Myers, M.W., and Hansel, W. (1995) The beneficial effects of incubating bovine embryos in groups are due to platelet-derived growth factor. *Theriogenology* **43**(1), 336-336

Thompson, J.G., Allen, N.W., McGowan, L.T., Bell, A.C., Lambert, M.G., and Tervit, H.R. (1998) Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. *Theriogenology* **49**(6), 1239-49

- Thompson, J.G., Mitchell, M., and Kind, K.L. (2007) Embryo culture and long-term consequences. *Reprod Fertil Dev* **19**(1), 43-52
- Trounson, A.O., Willadsen, S.M., and Rowson, L.E. (1977) Fertilization and development capability of bovine follicular oocytes matured in vitro and in vivo and transferred to the oviducts of rabbits and cows. *J Reprod Fertil* **51**(2), 321-7
- Vajta, G., Peura, T.T., Holm, P., Paldi, A., Greve, T., Trounson, A.O., and Callesen, H. (2000) New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. *Mol Reprod Dev* **55**(3), 256-64
- Van Langendonck, A., Donnay, I., Schuurbiers, N., Auquier, P., Carolan, C., Massip, A., and Dessy, F. (1997) Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. *J Reprod Fertil* **109**(1), 87-93
- van Soom, A., Ysebaert, M.T., and de Kruif, A. (1997) Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. *Mol Reprod Dev* **47**(1), 47-56
- Vutyavanich, T., Saeng-Anan, U., Sirisukkasem, S., and Piromlertamorn, W. (2011) Effect of embryo density and microdrop volume on the blastocyst development of mouse two-cell embryos. *Fertil Steril* **95**(4), 1435-9
- Walker, S.K., Heard, T.M., and Seamark, R.F. (1992) In vitro Culture of Sheep Embryos without Coculture - Successes and Perspectives. *Theriogenology* **37**(1), 111-126
- Wang, L.J., Xiong, X.R., Zhang, H., Li, Y.Y., Li, Q., Wang, Y.S., Xu, W.B., Hua, S., and Zhang, Y. (2012a) Defined media optimization for in vitro culture of bovine somatic cell nuclear transfer (SCNT) embryos. *Theriogenology* **78**(9), 2110-9
- Wang, Y.W., Ren, J.H., Xia, K., Wang, S.H., Yin, T.F., Xie, D.H., and Li, L.H. (2012b) Effect of mitomycin on normal dermal fibroblast and HaCat cell: an in vitro study. *J Zhejiang Univ Sci B* **13**(12), 997-1005
- Wheeler, M.B., Walters, E.M., and Beebe, D.J. (2007) Toward culture of single gametes: the development of microfluidic platforms for assisted reproduction. *Theriogenology* **68 Suppl 1**, S178-89
- Whitten, W.K. (1971) Nutritional requirements for the culture of preimplantation embryos in vitro. *Adv. Biosci.* **6**, 129-139

Wiley, L.M., Yamami, S., and Van Muyden, D. (1986) Effect of potassium concentration, type of protein supplement, and embryo density on mouse preimplantation development in vitro. *Fertil Steril* **45**(1), 111-9

Wolfenson, D., Roth, Z., and Meidan, R. (2000) Impaired reproduction in heat-stressed cattle: basic and applied aspects. *Anim Reprod Sci* **60-61**, 535-47

Wood, S.A., Allen, N.D., Rossant, J., Auerbach, A., and Nagy, A. (1993) Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* **365**(6441), 87-9

Wright, R.W., Jr., and Bondioli, K.R. (1981) Aspects of in vitro fertilization and embryo culture in domestic animals. *J Anim Sci* **53**(3), 702-29

Wydooghe, E., Heras, S., Dewulf, J., Piepers, S., Van den Abbeel, E., De Sutter, P., Vandaele, L., and Van Soom, A. (2013) Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reprod Fertil Dev*

Young, L.E., Fernandes, K., McEvoy, T.G., Butterwith, S.C., Gutierrez, C.G., Carolan, C., Broadbent, P.J., Robinson, J.J., Wilmut, I., and Sinclair, K.D. (2001) Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* **27**(2), 153-4

Young, L.E., Schnieke, A.E., McCreath, K.J., Wieckowski, S., Konfortova, G., Fernandes, K., Ptak, G., Kind, A.J., Wilmut, I., Loi, P., and Feil, R. (2003) Conservation of IGF2-H19 and IGF2R imprinting in sheep: effects of somatic cell nuclear transfer. *Mech Dev* **120**(12), 1433-42

Zhao, J., Whyte, J., and Prather, R.S. (2010) Effect of epigenetic regulation during swine embryogenesis and on cloning by nuclear transfer. *Cell Tissue Res* **341**(1), 13-21

Zuelke, K.A., and Brackett, B.G. (1990) Luteinizing hormone-enhanced in vitro maturation of bovine oocytes with and without protein supplementation. *Biol Reprod* **43**(5), 784-7

Appendix A – Media Formulations

Table A-1. Cell Culture Medium.

Stock	Final concentration
DMEM ^A	90% v/v
FBS ^A	10% v/v
Gentamicin ^B	50 µg/mL

Reagent sources A: HyClone B: Gibco

DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal Bovine Serum

Table A-2. Embryo Culture Medium.

Stock	Final Concentration (mM unless otherwise specified)
Water	
NaCl	107.7
NaHCO ₃	25.07
EAA ^A	2.8
NEAA ^A	1.0
Phenol Red	10 µg/mL
GlutaMAX ^B	1.00
KCl	7.16
CaCl ₂	1.71
Glucose	1.50
KH ₂ PO ₄	1.19
Gentamicin ^B	50 µg/mL
MgCl ₂	0.49
Lactate	3.30
Pyruvate	0.33
Bovine serum albumin ^C	8 mg/mL

Final pH: 7.2-7.4; mOsm 280 +/- 10. Reagents purchased from Sigma unless otherwise specified.
A:HyClone B: Gibco C: EMD Millipore

EAA: Essential Amino Acids; NEAA: Non-Essential Amino Acids

Table A-3. SOF-HEPES.

Stock	Final concentration (mM unless otherwise specified)
Water	
NaCl	107.7
Hepes	25.00
EAA ^A	2.8
NEAA ^A	1.0
Phenol Red	10 µg/mL
KCl	7.16
GlutaMAX ^B	1.00
NaHCO ₃	2.07
CaCl ₂	1.71
KH ₂ PO ₄	1.19
Gentamicin ^B	50 µg/mL
Pyruvate	0.33
MgCl ₂	0.49
Lactate	3.30
NaOH	0.35
Bovine Serum Albumin ^C	4 mg/mL

Final pH:7.2-7.4; mOsm: 280 +/- 10 Reagents obtained from Sigma unless otherwise specified.

A: HyClone B: Gibco C: EMD Millipore

EAA: Essential Amino Acids; NEAA: Non-Essential Amino Acids

Table A-4. Maturation Medium.

Stock	Final concentration
DMEM ^A	90% v/v
FBS ^A	10% v/v
FSH ^B	50 ng/mL
Gentamicin ^C	50 µg/mL

Reagent sources: A: HyClone B: Sioux Biochemicals C: Gibco

DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal Bovine Serum

Table A-5. Fertilization Medium.

Stock	Final Concentration (mM unless otherwise specified)
Water	
NaCl	107.7
NaHCO ₃	25.07
EAA ^A	2.8
NEAA ^A	1.0
Phenol Red	10 µg/mL
GlutaMAX ^B	1.00
KCl	7.16
CaCl ₂	1.71
KH ₂ PO ₄	1.19
Gentamicin ^B	50 µg/mL
MgCl ₂	0.49
Lactate	3.30
Pyruvate	0.33
Heparin	10 µg/mL
Bovine serum albumin ^C	8 mg/mL

Final pH: 7.2-7.4; mOsm 280 +/- 10. Reagents purchased from Sigma unless otherwise specified.

A:HyClone B: Gibco C: EMD Millipore

Appendix B – Permission Letters

5/20/2014

Rightslink Printable License

SPRINGER LICENSE TERMS AND CONDITIONS

May 20, 2014

This is a License Agreement between Bethany Gibson ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3393060140194
License date	May 20, 2014
Licensed content publisher	Springer
Licensed content publication	Journal of Assisted Reproduction and Genetics
Licensed content title	Effect of culture medium volume and embryo density on early mouse embryonic development: Tracking the development of the individual embryo
Licensed content author	Shan-Jun Dai
Licensed content date	Jan 1, 2012
Volume number	29
Issue number	7
Type of Use	Thesis/Dissertation
Portion	Figures
Author of this Springer article	No
Order reference number	None
Original figure numbers	Figure 1
Title of your thesis / dissertation	Improving development in in vitro produced bovine embryos cultured individually
Expected completion date	Jul 2014
Estimated size(pages)	75
Total	0.00 USD

Terms and Conditions

Introduction

The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).