A DYNAMIC CULTURE SYSTEM TO SUPPORT THE *IN VITRO* GROWTH AND MATURATION OF OVARIAN FOLLICLES

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Ovarian follicle growth is a prolonged process that involves progressive development of the follicle unit through specific histologically defined stages of development. Groups around the world have begun ovarian cryopreservation programs for young girls and women undergoing potentially sterilizing surgery or chemotherapy with the hope that follicles can be isolated from these tissues and grown *in vitro* at a later date. Though follicles derived from mice can be grown up to maturity using conventional culture techniques, scientists have been unsuccessful with the *in vitro* development of follicles from species that have larger follicles. The objective of this study was to develop a culture system that could better support the growth and maturation of these larger follicles. The aims of the study focused on maintaining structural integrity through a suspension culture technique, providing three-dimensional support by utilizing an alginate microencapsulation technique, and creating a unique oxygen environment that more closely mimicked the oxygen levels of the native ovary. The suspension culture technique was found to eliminate follicle flattening that occurred with larger follicles on flat surfaces in a static culture. The alginate microencapsulation technique was shown to improve the support of three-dimensional growth of preantral follicles; but requires the inclusion of FSH in the scaffold in order to maintain the growth rate of unencapsulated follicles. Finally, by implementing a dynamic oxygen protocol based on the unique oxygen environment of the ovary, both the yield and quality of the oocytes derived from *in vitro* cultured preantral follicles were significantly

improved when compared to oocytes from follicles cultured at the traditional ambient oxygen levels. In addition, these oocytes were not only able to undergo parthenogenetic activation, but were also fertilized through intracytoplasmic sperm injection. A subsequent gene expression analysis uncovered that follicles grown in a high oxygen environment possessed more differentially expressed genes compared to an *in vivo* control than did follicles cultured in a low oxygen environment. Furthermore, these differentially expressed genes were found to regulate several key processes that contribute to proper follicular development. These findings have contributed to the development of a novel culture system that has enhanced the *in vitro* support of follicle and oocyte maturation.

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PREFACE

I would like to start by thanking the Department of Bioengineering at the University of Pittsburgh, and its chair, Dr. Harvey Borovetz for his support and guidance. I would also like to thank the administrative personnel of the Department of Bioengineering, especially Ms. Lynette Spataro and Mrs. Joan Williamson for all of their help. I want to thank my dissertation committee for their suggestions and comments that truly enhanced my research. I would like to thank Dr. Elizabeth McGee for helping train me to be a scientist along with others at the Magee Womens Research Institute where I was given much assistance including Jill Brekoski. In addition, I'd like to thank Dr. Jaspel Khillan and the Transgenic Core for their expertise and the use of their equipment. I'd also like to thank Dr. Richard Koepsel who helped guide this project on a daily basis. Finally, I would like to thank Dr. Alan Russell for mentoring me, giving me the ability to become a skilled research scientist, and the opportunity to learn from someone who is adept at working within academia, industry, and government.

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1.0 INTRODUCTION

Therapeutic treatments for cancer and other diseases often adversely affect the reproductive cells of a patient. The very therapy that kills the cancer cells also kills the germ cells within the reproductive organs. For girls and women, this is a particularly harsh consequence because mammalian females are born with all the eggs they will ever have [1]. Should this original complement of eggs be destroyed, the result is sterility. For adult women, depletion of the supply of eggs results in menopause. Children whose eggs have been destroyed fail to undergo puberty. One step that can be taken to salvage some of the eggs is to remove and freeze the ovaries of girls and women prior to their undergoing potentially sterilizing therapies [2]. This banked tissue can then be used for transplant back into the patient to restore hormone and egg production. However, the major drawback with this procedure is that re-implanting the ovarian tissue carries the very real risk of transferring cancer cells that were resident in the tissue thus re-introducing the cancer as well [3]. This possibility could be avoided entirely by using the preserved tissue as a source of follicles that would be grown to maturity *in vitro*. This process could then be used as a source of mature oocytes for routine IVF procedures. The *in vitro* approach has here to fore been limited in that there is currently no technology to produce fertilizable eggs from the immature follicles present in the frozen ovaries of most mammals including humans.

Primordial follicles have a greater likelihood of surviving this cryopreservation [4]. Although primordial follicles derived from mice can be grown to maturity using static culture techniques [5-7], to date there have been no successful complete *in vitro* follicle development from larger species, such as rats, pigs and humans [8]. Intact rat, primate, or human preantral follicles have never been successfully cultured *in vitro* to maturation. Successful development of a technology in which preserved ovarian tissues can be utilized to restore fertility, would be a major development in reproductive science.

In addition to the potential advances in the area of infertility, the development of a successful follicle culture system can potentially become a more efficient source of matured oocytes for the derivation of embryonic stem cell lines. Recently, there has been a debate over the availability of the approximately 400,000 frozen embryos stored in IVF clinics across the United States for the production of new stem cell lines. However, of these 400,000 embryos, only 2.8% (~11,000) have been designated for research purposes. Using efficiency estimates for current technology, it is estimated that these 11,000 embryos would only successfully produce about 275 new stem cell lines [9]. Even this value is thought to be inflated because, more than likely, not all of the embryos earmarked for research will be exclusively used to created embryonic stem cell lines. Furthermore, the quality of these stored embryos is generally poor due to the fact that the high quality embryos are transferred to the patient during fertility treatments, leaving lower quality embryos stored in these banks [9].

Preliminary studies demonstrate that under conventional conditions, rat follicles frequently undergo flattening and rupture with loss of anatomic integrity that is important to the normal egg maturation process. When follicles were cultured in suspension culture systems, the follicles did not rupture and exhibited more robust growth. Furthermore, we discovered that by

utilizing a dynamic oxygen protocol in which the oxygen tension in the incubator is set to mimic the *in vivo* environment of the ovary, preantral rat follicles were matured to produce oocytes that resume meiosis and progress to the extrusion of the first polar body. Therefore, we believe that advances in bioreactor technologies hold great promise toward the development of a system that can support the complex needs of the developing follicle. This also could provide hope for young cancer patients to retain fertility. More generally, the problems associated with nurturing the development of the follicle mirror many of the unmet challenges in tissue engineering as a whole.

Our hypothesis is that follicle development and oocyte maturation will occur more efficiently in culture by providing metabolic support that more closely mimics the *in vivo* environment of the ovary.

The goal of this dissertation is to use a novel suspension culture system to support follicles into later stages of development. We propose that a suspension culture system can better support the metabolic and structural needs of the developing follicle. By optimizing the oxygen tension levels of the culture system and the three dimensional support of the follicle, we will answer basic biological questions regarding early follicle development and provide the basis for the prolonged growth of mammalian follicles and maturation of oocytes. Further, the use of such systems to generate embryonic stem cells could have far reaching effects on tissue engineering and regenerative medicine. Based upon these areas of research, the aims for the present study were as follows:

Specific Aim 1: Determine the effect of structural support, nutrient delivery, and mechanical shear stress on follicular growth and morphology. We hypothesized that, by

culturing follicles in a suspension culture system, the three-dimensional integrity of the follicular unit would be better supported. Furthermore, the utilization of alginate microcapsules could help maintain proper follicle morphology. In order to test this hypothesis, preantral ovarian follicles from Sprague Dawley rats were cultured in either of three different techniques, static culture in 96-well plates, orbiting test tubes, or rotating-wall vessels. Furthermore, additional follicles were cultured in suspension after being encapsulated in alginate microcapsules. Structural integrity was measured by the proportion of follicles that underwent follicular flattening, while morphology was assessed by histology and subsequent staining for gap junction proteins.

Specific Aim 2: Determine the effect of a dynamic oxygen delivery protocol that more closely mimicked the oxygen environment of the native ovary on oocyte viability and maturation. We hypothesized that the difficulty in achieving *in vitro* oocyte maturation may be due to the dysregulation of follicle development by exposure of early stage follicles to inappropriate oxygen concentrations. It is likely that *in vivo* follicles experience a transition from relative hypoxia in early development to the high volume of blood flow and oxygen delivery for pre-ovulatory follicles. This hypothesis was tested by culturing preantral follicles from Sprague Dawley rats in the orbiting test tube suspension culture system from aim 1 under two different oxygen regimens, ambient oxygen concentration throughout the duration of the culture or a dynamic oxygen environment in which the oxygen concentration was increased throughout the culture period. Oocyte viability was determined visually and oocyte maturation was determined by whether the oocyte was able to resume meiosis and undergo germinal vesicle breakdown. Oocyte maturation was further explored by comparing the efficiency of each group to produce oocytes capable of undergoing parthenogenetic activation.

Specific Aim 3: Determine the effect of oxygen concentration on preantral follicle gene expression. We hypothesized that by placing the follicles in the ambient oxygen group in an environment more suitable to mature graafian follicles, the granulosa cells may not differentiate properly, leading to desynchronized follicle development. This hypothesis was tested by culturing preantral follicles from Sprague Dawley rats for 3 days at two different oxygen concentrations, 4% or 20%. The gene expression pattern for these two culture groups along with an *in vivo* control group were obtained from analyzing the mRNA of each group on whole rat genome expression beadchips. Differentiated genes were then analyzed to determine if any could play a role in altering proper preantral follicle development.

1.1 OVARIAN ANATOMY AND PHYSIOLOGY

In the human female, the ovaries lie in the posterior wall of the pelvis in depressions known as the ovarian fossa (Figure1). Each ovary measures approximately 4cm in length, 2cm in width, and 8mm in thickness [10].

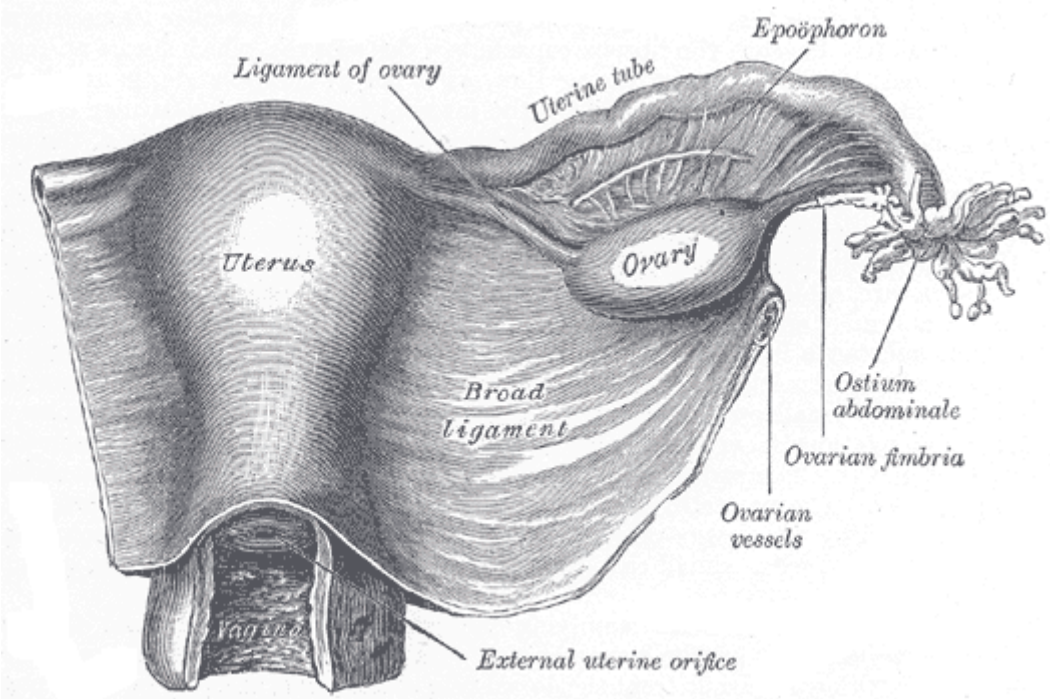


Figure 1: Uterus and right broad ligament, seen from behind. The broad ligament has been spread out and the ovary drawn downward. Reprinted from [10].

The outer surface of the organ is comprised of an epithelial cell layer known as the germinal epithelium of Waldeyer. Encapsulated by this epithelial layer is the ovarian stroma, which constitutes the bulk of the ovary. The stroma is divided into two regions, the cortex and the medulla (Figure 2). The external surface of the cortex is condensed, forming a thin layer of connective-tissue fibers known as the tunica albuginea. The stroma then transitions to a softer tissue composed of spindle-shaped cells as the connective tissue becomes less dense throughout the more interior regions of the cortex. The medulla is the most interior portion of the stroma. It is highly vascularized and forms the hilum, the tissue that attaches the ovary and through which the blood vessels enter [10].

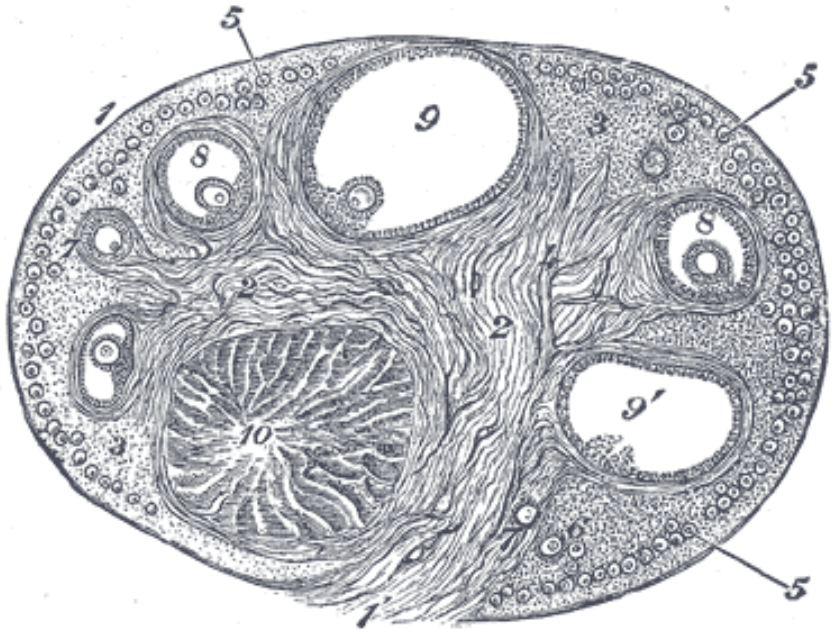


Figure 2: Section of the ovary. 1. Outer covering. 1'. Attached border. 2. Central stroma. 3. Peripheral stroma. 4. Blood vessels. 5. Vesicular follicles in their earliest stage. 6, 7, 8. More advanced follicles. 9. An almost mature follicle. 9'. Follicle from which the ovum has escaped. 10. Corpus luteum. Reprinted from [10].

The main functional unit of the ovary is the spherical follicle (Figure 3). In viewing a section of the ovary, the follicles are present throughout the entire stroma. The smaller immature follicles are located in the external cortical layer, while the more mature follicles increase in size as they move towards the highly vascularized medullary substance in the center of the ovary [10]. Throughout the cortical region, blood vessels are rarely observed in close proximity to any of the primordial or primary follicles [11]. In contrast, an abundance of blood vessels are found in the interior medullary region surrounding the secondary and antral follicles [11].

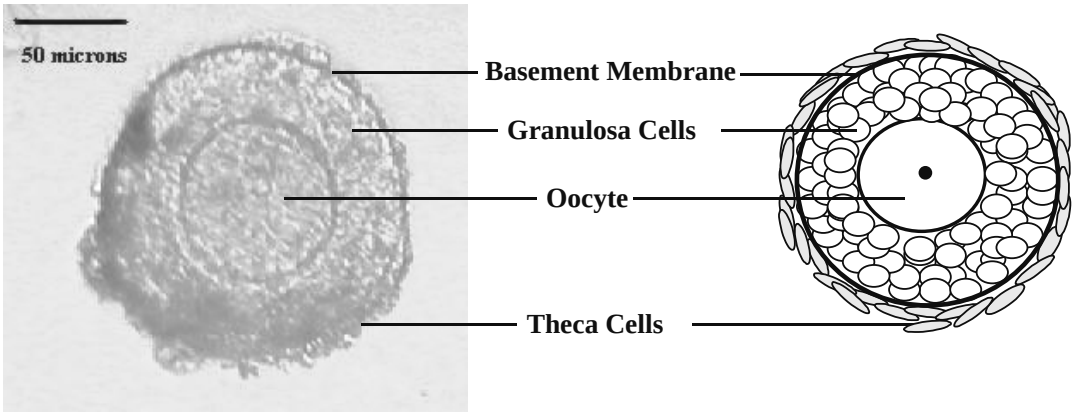


Figure 3: Anatomy of a Preantral Follicle.

Follicles consist of an oocyte surrounded by epithelial-like granulosa cells. The granulosa cells are surrounded by a basement membrane. Outside the basement membrane is a mesenchymal layer of cells called theca cells [1]. Follicles generally exist in a resting pool, in a relatively inert stage. An unknown mechanism results in the activation and growth of some follicles, while the majority of the follicle complement remains in the resting pool. When follicles are recruited from the resting pool, they grow independently through morphologically distinct stages known in order as, primordial, primary, secondary, preantral, antral, and Graafian [1].

Follicles develop through primordial, primary and secondary or preantral stages before acquiring an antral cavity, a fluid-filled cavity within the follicle. The fate of the vast majority of antral follicles is atresia, the degeneration of a follicle prior to maturity, via apoptosis [12]. Prior to puberty, follicles do not progress past the antral stage. At puberty the cyclic secretion of pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH) begins [1]. These hormones are necessary for the final stages of follicle growth and ovulation as well as stimulating the follicular estrogen production responsible for the physical changes of puberty.

After puberty, each month a few follicles are rescued from atresia by cyclic gonadotropins and go on to become pre-ovulatory follicles [1]. After a complex selection process, one of these follicles ovulates, releasing the egg, and transforms into a corpus luteum. Well over half of the time of folliculogenesis occurs prior to the antral stage of development. Unfortunately, knowledge about regulatory events during the prolonged developmental period of early follicles is very limited because it has not been possible to culture these early follicles over the prolonged course of development.

The oocytes develop within the ovaries through a process known as oogenesis. For humans, the germ cells have progressed from a primitive oogonia present throughout fetal development into what are known as primary oocytes at birth [10]. These primary oocytes possess the full compliment of 46 chromosomes and begin to replicate their DNA as they enter the first meiotic division. However, meiosis is arrested in the midst of prophase and the primary oocytes will remain in this state until puberty is reached [10].

At the onset of puberty, follicle stimulating hormone is produced and released by the pituitary. In response to FSH, several follicles are selected each month and begin to grow. As the follicles grow, factors are released that promote oocyte growth and development. One follicle will eventually become dominant over the other selected follicles and progress to become a graffian follicle. The oocyte contained within the maturing follicle is triggered to resume meiosis I (Figure 4). At this stage, the oocyte undergoes the first meiotic division, in which half of the chromosomes (46) go into a small cell known as the first polar body and the other half (46) stays within a larger cell that contains the vast majority of the cytoplasm and organelles from the original primary oocyte [10]. This larger cell is the secondary oocyte. Both of the resulting cells are diploid. The secondary oocyte then begins the second meiotic division, but

arrests during metaphase. At this point that ovulation occurs. The secondary oocyte will remain in this state until fertilization or the cell degenerates.

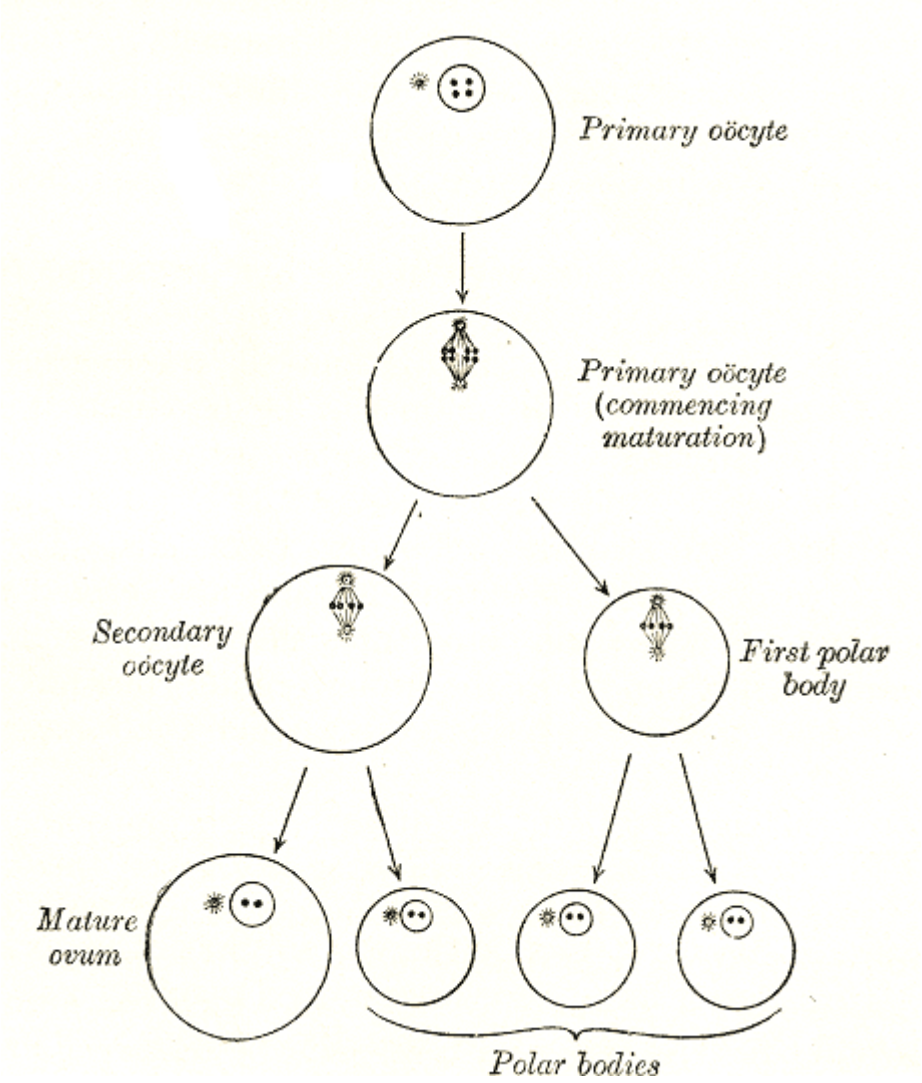


Figure 4: Diagram showing the reduction in number of the chromosomes in the process of maturation of the ovum. Reprinted from [10]

If fertilization is achieved, the secondary oocyte will resume meiosis II. Similar to the first division, the second meiotic division results in half the chromosomes (23) going into a second small polar body while the other half (23) remain in the much larger ovum with the cytoplasm and organelles [10]. This division results in two haploid cells, the second polar body and the ovum which will possess a maternal pronucleus containing 23 chromosomes (Figure 4).

Eventually, the paternal pronucleus from the sperm, also containing 23 chromosomes, will combine with the maternal pronucleus and form a zygote.

1.2 EFFECTS OF CHEMO AND RADIATION THERAPY

In the late 1970's, the 5-year survival rate for children treated for leukaemia was 33% [13]. By the early 1990's, that figure rose to 80% due to improvements in chemotherapy regimens and radiation therapy technology [14]. Thirty years ago, patients were lucky to be alive. Today, however, the outlook for young cancer patients is to have a normal reproductive life-span. As a result of this success, research is focusing on the need to address the significant morbidity associated with these treatments.

Chemotherapy refers to a type of treatment in which a chemical agent or a series of chemical agents are used to target cancerous cells. These chemical agents have the ability to inhibit cell division and cause cell death in dividing cells [13]. In the treatment of cancer, this is advantageous in that tumor cells are fast-dividing, so these drugs will in effect target these diseased cells. However, scientists have yet to develop a drug that specifically targets cancerous cells only. As a result, the chemotherapy agent can damage healthy cells as well, such as the fast-dividing cells within the hair follicles.

Radiation therapy refers to the use of ionizing radiation to manage malignant cells. In this method, an ion beam either directly or indirectly ionizes the atoms that comprise the DNA chain, thus damaging the DNA of the cell. In most cases, the ion beam ionizes water, forming free radicals, which will then damage the DNA. In the treatment of cancer, this technology is

used to both treat specific tumors and can be used for total body irradiation which prepares the patient for a bone marrow transplant [13].

Both treatments can result in ovarian toxicity. In the case of curative chemotherapy, germ-cell damage and ovarian failure is a common long-term side effect [13]. Studies have shown that older women are at greater risk of ovarian failure than younger women undergoing the same treatment [15, 16]. Furthermore, the class of chemotherapy agent can also affect the level of risk for ovarian tissue damage [13]. There are five classes of chemotherapy drugs: alkylating agents, aneuploidy inducers, topoisomerase II inhibitors, antimetabolites and radiomimetics. In most cases, a combination of these agents is used.

In the case of ovarian follicles, it was found that alkylating agents resulted in the greatest risk of causing ovarian failure [13]. These follicles, especially the primordial follicles, are quite susceptible to these chemotherapeutic agents. In an *in vitro* study with the chemotherapeutic agent cisplatin, pregranulosa cell swelling and pregranulosa cell nuclear swelling was observed in primordial follicles along with the disappearance of the follicle's lumen and oocyte [17]. Positive staining for apoptosis was also present within these cells. From these results, it was determined that chemotherapeutic agents reduce and possibly eliminate the follicle population in the ovary by inducing apoptosis in the pregranulosa cells due to the DNA damage caused by the agent in those cells [18].

A similar trend was discovered with radiation therapy. By directing an ion beam at a disease residing in the pelvis, such as cervical cancer, or the entire body, known as total body irradiation, the ovaries can be exposed to considerable doses of irradiation [17]. Studies have shown that 4 Gy is the approximate dose at which half of the ovarian follicles in a human will die [19]. Total body irradiation was found to cause ovarian failure in the majority of women at

10 Gy, 14.4 Gy, and using a fractionated 15 Gy protocol [20, 21]. Also, like with chemotherapy, the older the patient, the more susceptible their ovaries are to the treatment [21].

1.3 CRYOPRESERVATION OF OVARIAN TISSUE AND STRATEGIES FOR RESTORING FERTILITY

In response to the detrimental effects of chemotherapy and radiation therapy on the ovarian germ cells, researchers have worked to restore fertility through the cryopreservation of ovarian material for use after the cancer is successfully treated. There have been three cryopreservation strategies employed to address this problem; the cryopreservation of embryos [22], oocytes [23], and ovarian tissue [2, 24].

With embryo cryopreservation, patients first undergo hormonal ovarian stimulation usually before any potentially harmful cancer treatments are performed. The eggs are then collected and fertilized through a routine *in vitro* fertilization (IVF) procedure [25]. The resulting embryos are then cryogenically stored until needed.

Although this approach exclusively utilizes standard procedures with well defined protocol, there are a number of crucial disadvantages that make the development of an alternative method necessary. For instance, the ovarian stimulation procedure needed to procure the requisite number of mature eggs is inappropriate for prepubescent girls, eliminating this approach as an option for this group of patients [4]. For those women that this procedure is an option, some do not want to use donor sperm and have yet to find their mate. Furthermore, the time required to stimulate and mature the eggs is approximately 6 weeks [4]. In certain cancer patients, a delay in treatment this significant is undesirable. Also, it is theorized that the

hormone treatment used to stimulate the ovaries could augment the growth of oestrogen-sensitive tumors [4].

For these reasons, it became essential to explore the cryopreservation of the gametes instead of embryos in order to develop a more practical solution. One such method is the cryopreservation of a patient's oocytes. In this approach, mature oocytes could be stored until the patient wished to produce an offspring through IVF [23] or immature oocytes could be saved until such time they would be matured through *in vitro* maturation and fertilized through IVF [26].

For human oocytes, the development of a protocol to successfully and reliably cryopreserve mature eggs has yet to be discovered. Studies have found that the survival rate for freeze-thawed mature human oocytes is relatively low [27-29]. Furthermore, the fertilization rate for those oocytes that do survive the process is also quite poor [30]. In addition to the inefficient recovery and fertilization rates, the cryoprotective solutions have been found to damage the microtubules in the meiotic spindle apparatus which aligns the chromosomes in metaphase II [27]. The proper function of these microtubules is essential for the appropriate separation of the sister chromatids during the second meiotic division that will occur upon fertilization. Improper separation may result in an increased risk of chromosomal abnormalities.

In contrast, immature oocytes are more resistant to freeze-thaw damage due to their less differentiated state [4]. Compared to mature oocytes, immature oocytes have not yet progressed to metaphase II. In these oocytes, the microtubule spindle apparatus is disassembled and the chromatin is decondensed and still resides within a nuclear membrane, protecting these features from the cryopreservation process [4]. However, the drawback of storing these eggs is that, upon thawing, they must be matured in culture before fertilization can occur.

Studies have reported *in vitro* maturation rates that are highly variable and often inefficient [28, 31, 32]. It was also found that freeze-thawing oocytes prior to *in vitro* maturation significantly reduced the rate of blastocyst formation [32]. Until a more efficient method to mature oocytes *in vitro* is developed, this will not be a viable option for the preservation of fertility.

The third strategy is to cryopreserve sections of ovarian tissue. Originally, the goal of ovarian tissue cryopreservation was to regain endocrine function in overiectomized rodents. In the 1950's, researchers developed a protocol in which ovaries were frozen at -79 ˚C in a glycerol/saline solution [33-36]. After thawing, this tissue was autologously re-implanted subcutaneously in the animal. It was found that the resumption of oestrous cycles occurred 2-3 weeks post re-implantation. As a result of this success, they then attempted to restore fertility in these animals by re-implanting the tissue back in its original site. This protocol did produce viable offspring, however, the success rate was relatively low [37]. Although the technology was promising, the 1950's had few applications for it.

However, as cancer therapies improved, the positive outlook for young cancer patients became a catalyst for renewed interest in the technology. For this application, the preservation of ovarian cortex tissue was an ideal strategy. Not only can this tissue be retrieved by a less invasive laparoscopic surgery, but this region of the ovary is where the majority of the primordial follicles lie. This is crucial because it is the primordial follicles that are most likely to survive the cryopreservation due to their small size and the undifferentiated state of their cells [4].

Since the 1950's, there had also been major advances in cryopreservation technology with the advent of the controlled rate freezing apparatus and the development of more effective cryoprotective agents. It was now understood that follicle survival depended on the efficiency of

the cryoprotective agent to enter the tissue in order to protect the follicle from freezing damage. It was found that agents such as dimethyl sulphoxide, propylene glycol, and ethylene glycol significantly increased follicle freeze-thaw survival rates when compared to the previously used glycerol supplemented medium (Figure 5) [38]. It is believed that the higher viscosity of the glycerol based mediums caused lower permeation rates, resulting in a less effective protective agent. Utilizing this new generation of cryoprotective agents, studies have shown that ovariectomized mice implanted with autographed frozen-thawed ovarian tissue demonstrated the resumption of oestrous cycles in 75% of the animals and pregnancies were reported in 73% of recipients [39].

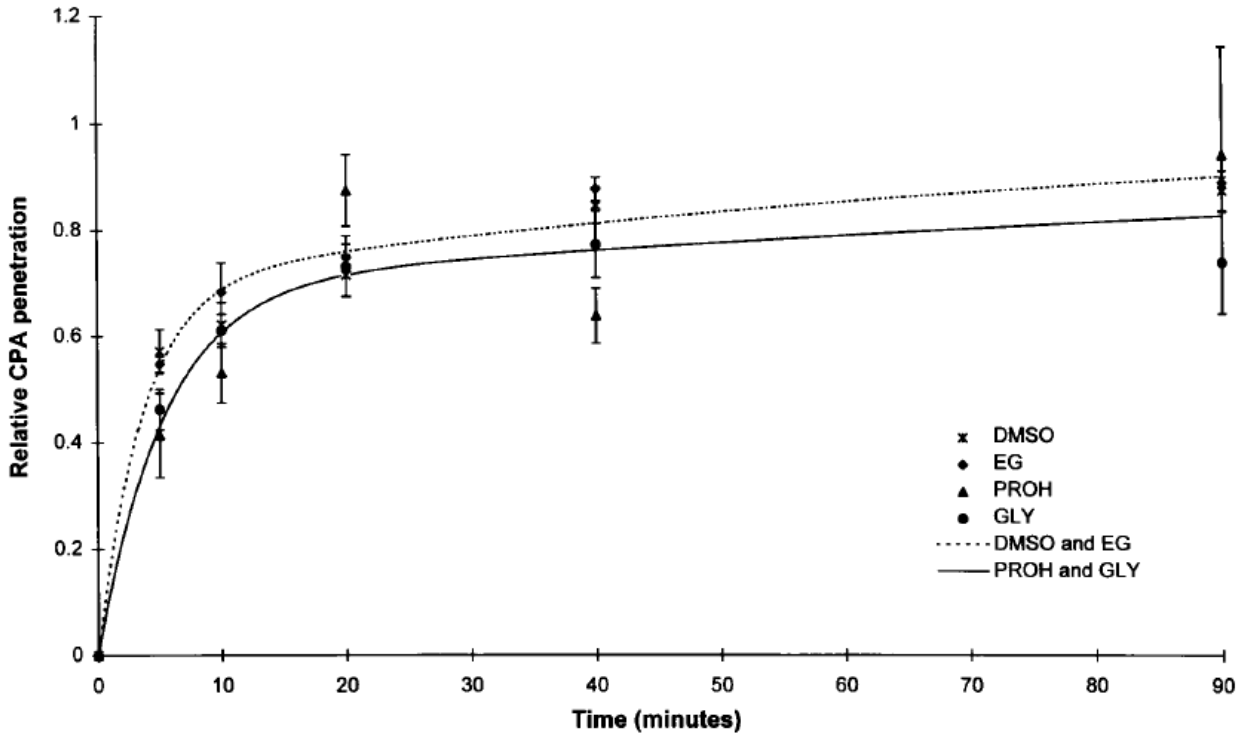


Figure 5: The permeation rates of cryoprotective agents: dimethyl sulfoxide (DMSO), ethylene glycol (EG) propylene glycol (PROH), and glycerol (GLY). Reprinted from [38]

For the purpose of restoring fertility in humans, researchers have developed three broad strategies to produce mature eggs from cryopreserved ovarian tissue, autologous re-implantation, xenotransplantation into an immunodeficient animal, and the *in vitro* culture of follicles isolated from the ovarian tissue (Figure 6). Of these methods, only the autologous re-implantation of the patient's banked ovarian tissue either at the orthotopic site or a heterotopic site will serve to restore fertility and endocrine function, thus eliminating the need for hormone replacement therapy [40]. At the orthotopic site, the patient's cortical biopsies are sutured into a resected ovary prepared laparoscopically prior to the procedure. Not only will this reestablish the patient's endocrine function, but also restores their 'natural' fertility, as matured eggs will be released into the fallopian tubes [4]. At a heterotopic site such as the abdominal wall, the re-implanted tissue would need to be hormonally stimulated and aspirated in order to collect the mature eggs and fertilization would then require IVF [41].

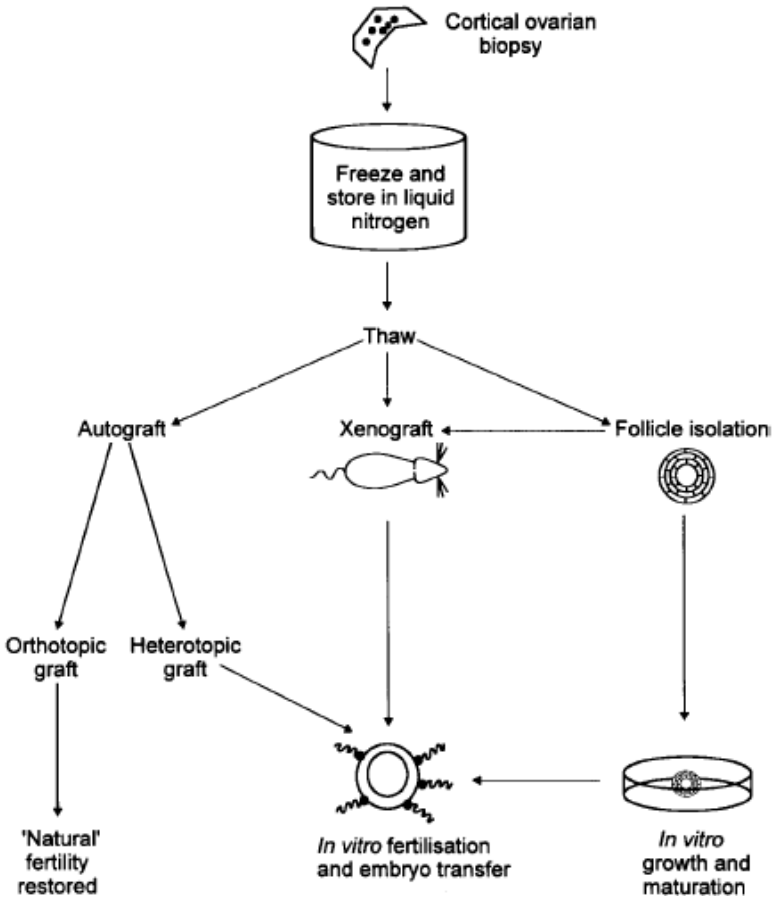


Figure 6: Strategies for the restoration of fertility using cryopreserved ovarian tissue. Reprinted from [4]

Although this strategy has the advantage of regaining endocrine function, there are significant drawbacks with the procedure. First, it is unknown how long ovarian function will last. Ovarian function is lost when the follicle population dips below ~ 1,000 [42]. Furthermore, it has been found that the follicle depletion rate increases once the total number of follicles hits 25,000 [42]. Regardless of whether the follicle survival rate is high in the thawed tissue, the follicle population in the cortical biopsies is still relatively low. Therefore, it is likely that ovarian function would be restored for only a short duration.

Second, re-implanting tissue from cancer patients runs the added risk that the biopsies would contain malignant cells [43, 44]. This especially is the case for patients with blood-borne

cancers like leukaemia or cancers that are known to attack the ovaries. This has been shown in the murine model in which healthy recipients of frozen-thawed ovarian tissue from donors with lymphoma had an 85% mortality rate from the transferred malignant disease [3].

Xenotransplantation of the ovarian tissue is a strategy that would be less invasive to the patient and still provide a vascularized *in vivo* environment to support the maturation of the follicles [45]. With this approach, cortical biopsies are implanted into hypogonadal immunodeficient mice. Hormones are administered exogenously to stimulate follicle growth. When the eggs are mature, follicles would be aspirated and the eggs retrieved and fertilized through IVF [45]. However, due to ethical and moral issues, this procedure is impractical for human application. Instead, this approach is being explored for use in endangered species conservation [4].

The final strategy is the *in vitro* culture of isolated follicles from the frozen-thawed ovarian tissue. In this method, follicles are isolated via manual dissection with syringe needles or enzymatic digestion. The follicles are then placed in culture medium supplemented with FSH and a variety of additional components. Within this strategy there are many culture techniques aimed at maturing the follicles. Researchers have designed techniques that grow follicles on a surface, within a microcapsule, and in suspension. An overview of the different techniques and a brief history of the field are outlined in Chapter 1.5.

One of the advantages of this approach is that, by isolating the follicles, it allows any malignant cells present within the cortical biopsy to be removed. Furthermore, by maturing the oocytes *in vitro*, it eliminates any invasive surgical grafting procedures.

1.4 AUTOLOGOUS RE-IMPLANTATION OF CRYOPRESERVED OVARIAN TISSUE

Although there are a variety of approaches to cryopreserving ovarian tissue for transplantation back into the patient, the method that has been utilized exclusively in clinical trials to this point has been the storage and re-implantation of avascular cortical biopsies [46]. This remains the only procedure in humans that has resulted in viable offspring [47]. Currently, there have been 17 reported attempts to regain endocrine function and fertility through the autotransplantation of cryopreserved ovarian tissue at either an orthotopic site (8 cases), a heterotopic site (6 cases), or both an orthotopic and a heterotopic site (3 cases). Table 1 outlines the results of 15 of the trials [46].

Of the 8 cases in which only an orthotopic site was utilized, 3 resulted in viable pregnancies and corresponding live births. The first successful live birth from cryopreserved ovarian tissue was reported in Donnez et al. (2004) [47]. In this case, the patient's stored tissue was thawed and transplanted laproscopically into a pocket surrounding the patient's ovary created a week prior to the surgery. After 5 months, the patient resumed regular ovulatory cycles for 4 months, engaging in natural intercourse, at which point a viable intrauterine pregnancy was confirmed. This pregnancy eventually resulted in a live birth. Further data, such as whether this patient resumed normal ovarian function after the pregnancy, how long it remained, and if the cancer returned, has not been reported.

The second birth was reported a year later in Meirow et al. 2005 [48]. Similar to the previous case, the ovarian tissue sections were surgically transplanted at the site of the native ovary. However, in this trial, mature oocytes were collected following hormone injections and follicle aspiration. Using this method, one metaphase II oocyte was retrieved and fertilized via

intracytoplasmic sperm injection. Once it reached the 4-cell stage, the embryo was transferred to the patient's uterus, resulting in a viable pregnancy and a live birth.

The final case of a live birth was reported from the same group in a manuscript not listed in Table 1, Meirow et al. (2007) [49]. The procedure used was identical to their 2005 case except that fertilization took place *in vitro* without the need of intracytoplasmic sperm injection. These remain the only three cases that claim a live birth originating from an oocyte from the cryopreserved ovarian tissue.

The remaining 14 cases report results with varying degrees of success in terms of regaining endocrine function, retrieving mature oocytes, producing embryos, and inducing pregnancies. With the exception of Oktay (2006) [50], none of the other trials result in a live birth. Furthermore, it was determined that the live birth reported in Oktay (2006) did not originate from the transplanted ovarian tissue, but instead was produced by the native ovary.

Table 1: Autotransplantation of Cryopreserved Human Ovarian Tissue. Reprinted from [46]

Reference	Age before freezing	Chemo before freezing	Indication	Graft site	Graft size	Recovery of ovarian function	Outcome
Oktay and Karlikaya (2000)	29	No	Intractable menometrorrhagia	Orthotopic: pelvic peritoneum	8 pc (5–10 × 2 mm)	16 weeks	↑ E_2, FD after stimulation, ovulation, menses
Radford *et al.* (2001)	36	Yes	Hodgkin's lymphoma	Orthotopic: ovarian	2 pc (10 × 5 × 1 mm)	8 months	↓ FSH and LH, ↑ E_2, FD, ovulation, menses (1 cycle)
Callejo *et al.* (2001)	47	No	Uterine leiomyoma	Heterotopic: rectus abdominis muscle	40–45 pc (2–3 mm^3)	3–4 months	↑ E_2, FD after stimulation (follicle of 20 mm)
Kim *et al.* (2004a)	37	No	Squamous cell cervical carcinoma (Ib)	Heterotopic: breast [between breast tissue and pectoralis muscle (a)] + abdominal [rectus muscle (b)]	40 pc (5 × 5 × 1–2 mm): 20 (a) + 20 (b)	14 weeks	↑ E_2, ↓ FSH, FD (follicle of 11–16 mm) only in (b), ovulation
Oktay *et al.* (2004)	30	No	Breast cancer	Heterotopic: s.c. abdominal wall	15 pc (from 5 × 5 × 1 to 15 × 5 × 2 mm)	3 months	FD after gonadotrophin stimulation, oocyte retrieval, 20 oocytes, 8 IVF–ICSI, 4-cell embryo transfer + 1 aneuploidic embryo
Donnez *et al.* (2004)	25	No	Hodgkin's lymphoma	Orthotopic: ovarian fossa peritoneum	1 pc (12 × 4 × 1 mm) + 67 (35 + 32) pc (2 × 2 × 1 mm)	$4^1/_2$ months	↑ E_2, ↓ FSH and LH, FD (follicle of 22 mm), regular cycles (± 5 weeks), pregnancy, live birth
Meirow *et al.* (2005)	28	Yes	Non-Hodgkin's lymphoma	Orthotopic: ovarian	3 pc (15 × 5 × 1–2 mm) + tiny fragments injected	8 months	↓ FSH, ↑AMH and inhibin B, FD, ovulation, menses, modified natural cycle, oocyte retrieval, 1 metaphase II oocyte IVF–ICSI, 4-cell embryo transfer, pregnancy and live birth
Schmidt *et al.* (2005)	28	No	Hodgkin's lymphoma	Orthotopic: ovarian (a) + peritoneum (b) and heterotopic: abdominal wall (c)	12 pc: 4 (a) + 4 (b) + 4 (c)	19/22 weeks	↑ E_2, ↓ FSH, FD (19 weeks), menses (22 weeks), no stimulation, empty follicle (of 20 mm)
	25	No	Hodgkin's lymphoma	Orthotopic: ovarian (a) + peritoneum (b) and heterotopic: abdominal wall (c)	12 pc: 4 (a) + 4 (b) + 4 (c)	18/25 weeks	↑ E_2 ↓ FSH, FD (18 weeks), menses (25 weeks), 2 IVF cycles, 2 metaphase II oocytes, 2-cell embryo
	32	No	Non-Hodgkin's lymphoma	Orthotopic: ovarian	6 pc	8/14 weeks	↑ E_2, ↓ FSH, FD (8 weeks), menses (14 weeks), 1 IVF cycle, 1 metaphase II + 1 GV oocyte, 4-cell embryo
Wolner-Hanssen *et al.* (2005)	30	No	Pure red cell aplasia + BMT	Heterotopic: s.c. forearm	10 pc (1–2 × 1–2 × 0.5–1 mm)	18 weeks	FD (12.6 mm follicle and 6.7 mm follicle) after local gonadotrophin stimulation
Donnez *et al.* (2006)	21	No	Sickle cell anaemia + BMT	Orthotopic: ovarian (a) + ovarian fossa peritoneum (b)	69 pc (2 × 2 × 1 mm): 45 (a) + 24 (b)	$4^1/_2$ months	↑ E_2, ↓ FSH and LH, FD (follicle of 20 mm), menses
Oktay (2006)	29	Yes	Hodgkin's lymphoma	Heterotopic: s.c. abdominal wall	Left ovary in pieces	2 months	↑ E_2, FD, ovulation, 6-week pregnancy and miscarriage, and pregnancy with live birth from native ovary
Demeestere *et al.* (2006)	24	Yes	Hodgkin's lymphoma	Orthotopic: ovarian (a) + ovarian fossa peritoneum (b) and heterotopic: s.c. abdominal wall (c)	18 pc (5 × 5 × 2 mm): 3 (a) + 9 (b) + 6 (c)	4 months	↑ inhibin B and E_2, ↓ FSH, FD in all sites: (a) large follicles in ovarian site, (b) only one dominant follicle and (c) follicles <13 mm, 6 ovulations, natural conception, pregnancy, miscarriage at 7 weeks (aneuploidy)
Donnez *et al.* (unpublished data)	24	Yes	Non-Hodgkin's lymphoma	Orthotopic: ovarian	5 pc (10 × 4–5 × 1 mm)	5 months	↑ E_2, ↓ FSH and LH, FD (follicle of 21 mm), menses

AMH, anti-Müllerian hormones; BMT, bone marrow transplantation; E_2, estradiol; FD, follicular development; GV, germinal vesicle; pc, pieces.

As promising as these results are, there are several concerns and questions pertaining to this approach. The first of which questions the validity of the three live births. In all three cases, the source of the fertilized oocyte is not entirely clear. Each manuscript claims that the oocyte originated from the transplanted cryopreserved ovarian tissue. However, the site of the transplant in all three trials was the ovarian cortex or ovarian fossa peritoneum. Therefore, the chance that the oocyte was actually derived from the surrounding native ovarian tissue and not the transplanted ovarian tissue cannot be dismissed.

This concern is further validated with a more in depth analysis of the pregnancy and live birth reported in Oktay (2006) [50]. With the transplant location being the abdominal wall, a natural pregnancy from that tissue is impossible. The patient in this study experienced recurrent pregnancies, the first resulting in a miscarriage and the second producing the live offspring. The only source for these oocytes was one of the menopausal ovaries. The mechanism behind the spontaneous recovery of ovarian function is unknown. However, a controversial theory has been debated in recent years, which suggests that ovarian tissue transplantation may trigger the spontaneous resumption of ovarian function within the native ovary. Ongoing studies are testing the hypothesis, but if this is possible, it would be difficult to conclude with absolute certainty that an orthotopic site transplant produced the fertilized oocytes. Furthermore, this would add another factor to the efficacy of the procedure, the degree of damage to the germ cells caused by the chemotherapy or radiation therapy.

A second concern raised from analyzing this data is the high variability in terms of both the length of time before the patient regains ovarian function after the transplantation and the duration that this function is sustained. When comparing the Donnez et al. (2004) [47] and Radford et al. (2001) [51] reports, the patients suffered identical cancers and were transplanted at

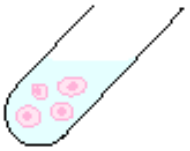
the same site. While the 2004 patient went on to have 5 cycles starting 4.5 months after the surgery, the 2001 patient had 1 cycle that occurred 8 months after transplantation. It should be noted that the patients' age difference could account for the delay and minimal recovery observed in the older patient. However, this suggests that there will be limitations for older patients and that this procedure may require a significant amount of tissue at a time when the patient will not know if the therapy will even affect their germ cells.

Finally, almost all of these studies report on a single patient with some degree of success. Until there is a more robust clinical trial that can produce a sample population greater than n=1, the true efficiency of this strategy will remain unknown.

1.5 CONVENTIONAL *IN VITRO* OVARIAN FOLLICLE CULTURE TECHNIQUES

For three decades, researchers have been attempting to develop a long-term culture technique that could support the maturation of oocytes from early stage follicles. Although this research has greatly increased our knowledge and understanding of follicular and oocyte development, the creation of viable offspring from oocytes recovered from *in vitro* cultured preantral follicles has only occurred in the mouse [52]. Table 2 outlines the various techniques developed over the years [53]. This section will provide a brief history of this technology.

Table 2: Various Approaches to Follicle Culture. Reprinted from [53]

Method		Duration	Examples of uses	Species	Example references
Organ culture (+/– perifusion or perfusion)		Usually short because of necrosis unless ovary is very small	Blood flow effects on ovulation, culture of immature whole ovaries	Rabbit, rat, mouse	Bonello *et al.*, 1996; Eppig and O'Brien, 1996
Short-term		<72 h	Steroid production, endocrine responses. (Usually large follicles.)	Hamster, sheep, rabbit	Kitzmann and Hutz, 1992; Tambe and Nandedkar, 1993; Thébault *et al.*, 1983
Group culture	Gel	~7 days	Observation of follicle growth, endocrine studies, preparation for transplantation	Mouse, human, cattle	Carroll *et al.*, 1991a; Roy and Treacy, 1993; Hulshof *et al.*, 1995
	Contact	6–12 days	Interfollicular communication	Mouse	Qvist *et al.*, 1990; Spears *et al.*, 1996
	No contact 		Interfollicular communication	Mouse	Spears *et al.*, 1996
	Non-spherical 	7–9 days	Production of large numbers of oocytes, follicular endocrinology	Mouse, rat	Eppig, 1992; Cain *et al.*, 1995
Individual culture	Spherical 	4–16 days	Follicle metabolism, steroidogenesis and oocyte development	Mouse, rat, pig	Boland *et al.*, 1993; Hartshorne *et al.*, 1994a; Hirao *et al.*, 1994; Smythe *et al.*, 1994
	Non-spherical 	10–16 days	Hormonal influences, oocyte development	Mouse	Cortvindt *et al.*, 1996, in press; Smitz *et al.*, in press.

1.5.1 Follicle Culture Techniques

Whole organ culture of the ovary is reserved mostly for small rodents. It allows for the follicles to remain in their native environment, helping maintain three dimensional structure and basement membrane integrity as it interacts with the surrounding extracellular matrix [54]. However, this approach can only be utilized in short-term cultures as necrosis will occur throughout the medulla region due to a lack of oxygen and nutrient delivery. Only the follicles located in the outside cortical region of the ovary will be able to survive.

Although this method cannot fully support the growth of a mature follicle, it has been used to initiate the activation of primordial follicles. The initial studies cultured entire ovaries from newborn mice for 8 days, when it was observed that several primordial follicles had progressed to secondary preantral follicles [54]. At that point, those follicles were isolated and grown individually and eventually produced fertilizable oocytes. For larger species, the whole organ culture is unrealistic, but work continues on using a similar two-step system with ovarian tissue strips.

Another attempt at multiple follicle culturing involves a collection of isolated follicles grown within a gel or on top of a surface. If given the freedom to move, follicles will have a tendency to aggregate and grow into one another.

Individual follicle culture also occurs within gels or on a surface. Preantral mouse follicles will remain spherical and fully mature in a period of approximately 6 days on a tissue culture treated surface [52]. The resulting oocytes can be fertilized and have produced viable pups. These results have also been obtained by encapsulating individual mouse follicles in hydrogel microcapsules [7].

Typically, individual murine follicle cultures will result in roughly 20 % of the follicles dying due to physical damage attributed to the isolation technique and a small proportion of the remaining follicles undergoing atresia [52, 55]. However, the percentage of follicles that respond to the culture and progress through the antral transition, approximately 40-75%, significantly surpasses the proportion that reaches this stage *in vivo*, about 20% [56].

Although this technique eliminates the necrosis associated with the multiple follicle approaches, it has the disadvantage of the follicles flattening out onto the surface they are being cultured upon. This has been found to effect cell-to-cell junctions and causes the cells that plate down to alter their morphology [57].

1.5.2 Follicle Culture Media

Traditionally, ovarian follicles are cultured in alpha modified minimal essential medium due to its ability to properly support rapidly dividing cell types [53]. This is then accompanied with a protein source, such as fetal bovine serum (~5%). Serum supplies various proteins that support follicle growth and survival [58]. However, if there is a desire to use a fully defined media, insulin, transferrin, and selenium can be substituted [59]. Insulin has been determined to have multiple roles during early follicle development. For preantral follicles, insulin has been found to be crucial for follicle survival, increase oocyte growth, and help trigger granulosa cell differentiation [60, 61].

In addition to these components, a number of hormones are also added to the media in a time sensitive manner such as follicle stimulatin hormone (FSH), human chorionic gonadotropin (hCG), and luteinizing hormone (LH). It is known that *in vivo* follicles transition from the primordial to the secondary preantral stage without the influence of FSH. However, it has been

shown that preantral granulosa cells possess FSH receptors. Further studies confirmed that FSH not only promotes granulosa cell proliferation and differentiation, but also decreases the proportion of follicles that undergo atresia *in vitro* [62-64]. For late preantral and antral follicles, continued growth is entirely dependent on FSH. Then, just prior to ovulation, LH or hCG can be administered to trigger ovulation. If given to early, LH and hCG can hinder follicular growth and alter the three dimensional structure of the follicle [65].

In addition to these effects, gonadotrophins appear to help ease oxidative stress on follicles *in vitro* [66]. *In vitro*, it is likely that oxidative stress and free radical damage is increased due to the incubator air being conditioned to 20% oxygen instead of the approximately 5% experienced *in vivo* [53]. This oxidative stress initiates factors within the follicle that promotes atresia. Gonadotrophins seem to lessen these effects by triggering scavenger systems within the follicle to maintain viability [66]. However, this damage can be eliminated by culturing these follicles at more appropriate oxygen tensions.

2.0 SUSPENSION CULTURE

2.1 INTRODUCTION

A young female patient undergoing chemo or radiation therapy to treat cancer is often sacrificing her fertility and hormonal development, since these treatments can also result in the loss of ovarian germ cells, or eggs. The loss of eggs within the ovary renders a woman sterile, but the loss of the follicular unit surrounding the egg results in the inability of the ovary to produce the hormone estrogen. Although it is possible for ovaries to be removed and then frozen prior to cancer treatment, there is currently no reliable *in vitro* technique that can subsequently produce fertilizable eggs upon thawing of this stored tissue [6, 67]

Eggs, or oocytes, are located in ovarian follicles, the main functional unit of the ovary. Once formed, follicles remain dormant in a resting pool until an unknown mechanism activates them for growth [6]. Over time follicles are gradually introduced into the growing pool until the resting pool is depleted and menopause ensues. When recruited from the resting pool, follicles spend most of their time developing through primordial, primary, and secondary stages [1]. Prior to puberty, secondary follicles develop up to the antral stage, but then undergo apoptosis. After puberty, however, a cohort of follicles is rescued from apoptosis each month by the cyclic secretion of pituitary Follicle Stimulating Hormone (FSH) [1].

Although much is known about the development of late-stage follicles, relatively little has been reported on the development of follicles prior to the antral stage [6, 67].[i] In addition to the oocyte, which is innermost, a preantral follicle is comprised of epithelial-like granulosa cells, which are surrounded by a basement membrane, and theca cells, which are mesenchymal cells that surround the exterior side of the basement membrane (Figure 3). Preantral follicles grow at a slow rate and their small size makes them more difficult to isolate for culture [67]. Due to complex follicle structure and tightly controlled dynamics of follicle maturation, maintaining optimal follicle growth and morphology *in vitro* is difficult. Interestingly, follicle flattening and rupture [67-70] often occurs around 48 to 72 hours of static culture in a tissue culture well (Figure 7). Follicle rupture is characterized by the disruption of the follicle and the movement of the innermost granulosa cells and oocyte through a defect in the basement membrane. Although the granulosa cells can remain healthy after follicle rupture, the normal three-dimensional relationship of the follicle has been lost and oocyte maturation may not progress normally.

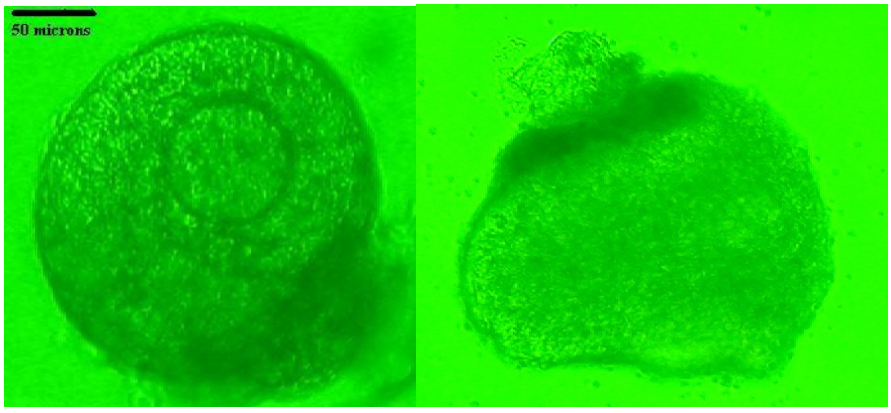


Figure 7: Flattened and Ruptured Rat Preantral Follicles. ***Left:*** Flattened; *Right:* Ruptured**.**

Follicle flattening and rupture could be the result of a non-uniform environment of a flat surface, nutrient transfer limitations, or a combination of the two. In the ovary, follicles are spherical and appear uniformly supported since they are located within the ovarian stroma, which is rich in extracellular matrix support. Although there are various approaches to follicle culture [53, 67, 70] follicles are conventionally cultured in tissue culture wells. As a follicle rests on the surface of a well, it is possible that the dispersion of forces within the follicle become imbalanced, resulting in a loss of architecture and eventually a discrete rupture of the basement membrane. During conventional culture, there is no agitation to prevent a follicle from settling to the surface of the well. Therefore it is also likely that nutrient delivery from the media to the follicle is sub-optimal since nutrient diffusion is hindered on the bottom of the follicle, limiting the surface area exposed to the nutrient rich media.

In the study described herein, we examine the impact of suspension culture systems, which provide a more uniform environment to the follicle, on the degree of flattening and rupture. The suspension culture systems used in this study include orbiting test tubes and custom designed NASA-type rotating-wall vessels. The test tubes are positioned in an orbiting device that gently agitates the media just enough to prevent a follicle from settling to a surface. The rotating-wall vessels are cylindrical chambers that axially rotate the media, thereby preventing a follicle from settling to a surface.

Ovarian follicles are extremely fragile, and mechanical damage may be experienced due to shearing. Semipermeable hydrogels have been used to encapsulate tissue, functioning as a barrier from infection and mechanical stress [1, 67]. Alginate is one of the most commonly applied biomaterials for microencapsulation due to its biocompatibility, high affinity to water, and ability to form gels under mild conditions when in the presence of calcium ions [1, 12, 67,

69, 70]. Alginate is comprised of chains of alternating blocks of mannuronic acid (M), which contributes the elastic property of the gel, and guluronic acid (G), which contributes mechanical strength, stability, porosity, and gel forming properties [67, 71]. Alginates are extracted from all species of brown algae and contain differing compositions of MM, MG, and GG blocks offering a variation in strength and stability [72]. The high porosity range of a 2 % Ca^{2+} alginate gel bead, 5 nm to 200 nm in diameter, only limits the diffusion of large proteins, and thus may be beneficial for culturing immobilized whole cells, cell aggregates, or tissue [73]. It has been reported that substrates of molecular weight less than 2×10^4, such as glucose, L-tryptophan, and α-lactoalbumin, are able to diffuse freely into and from calcium alginate beads at approximately the same diffusion rate as in water, [67] while there is some resistance for large proteins of molecular weight greater than 3×10^5 [73]. Therefore, follicles imbedded in alginate beads may be protected from mechanical shear stress in the suspension culture systems while maintaining nutrient delivery and providing three-dimensional structural support.

In this study, we evaluate the impact of culturing non-encapsulated and encapsulated rat and mouse preantral follicles by the conventional culture system and suspension culture systems designed to provide uniform structural support and enhance nutrient delivery. We investigate what role structural support, nutrient delivery, and mechanical shear stress may have on follicular growth and morphology.

2.2 MATERIALS AND METHODS

2.2.1 Culture Preparation

Culture media consisted of α–Minimal Essential Medium (Gibco BRL, Invitrogen Corporation, Grand Island, NY) with additives of 8-bromo-cGMP (5 mM), ITS+ (1 % solution of insulin, 10 mg/L; transferrin, 5.5 mg/L; linoleic acid, 4.7 mg/L; selenium, 5 mg/L), Pen/Strep (1 %, penicillin 100 U/ml, streptomycin 100 μg/ml), all from Sigma Chemical Co. (St. Louis, MO), and recombinant Follicle Stimulating Hormone, rFSH, (100 ng/ml; Serono Laboratories, Geneva). As a control group, FSH was deleted from the media of some culture wells, since it is added to induce follicle growth [1, 67]. Once prepared, culture media was placed into culture wells (150 μl/well), test tubes (500 μl/tube), and rotating-wall vessels (4000 μl/vessel) and then incubated to maintain conditions of 5 % CO_2 and 37 ^{0}C.

Orbital culture was provided by placing the 6 ml culture tubes in a circular rotator plate (Glas-Col, Terre Haute, IN), having a diameter of 30.5 cm, which was rotated around its horizontal axis at rate between 8-15 rpm. Therefore, as the plate rotates, the tubes slowly orbit the axis of the plate. Rotational culture was achieved with the use of rotating-wall vessels modeled after the NASA design. These reactors were constructed in house and each cylindrical vessel has a diameter of 2.5 cm, a width of 0.6 cm, and a motor that rotates the vessel axially at a rate between 8-15 rpm.

2.2.2 Ovarian Excision

Ovaries were excised from euthanized female Sprague-Dawley rats or C56BL/6 mice between the ages of 12 to 20 days. Animals were maintained in a certified animal care facility according to approved institutional guidelines. The ovaries were dissected clean and immediately placed in warmed dissection medium consisting of Leibovitz L-15 Medium (Gibco BRL) with 1 % bovine serum albumin.

2.2.3 Follicle Isolation and Culture

Follicles were mechanically dissected from the ovaries under a dissecting microscope using 25 gauge needles. Follicles of 140 μm to 150 μm in diameter were selected and measured using an inverted Zeiss microscope equipped with an ocular micrometer. Follicle diameter was measured as the distance between the inner edges of the basement membrane. Individual follicles were then immediately transferred to culture wells, test tubes, or rotating-wall vessels and placed in a CO_2 incubator for 72 hours. Follicle diameter was measured daily to evaluate the impact of orbital and rotational culture on growth. For follicles that appeared flattened, the longest diameter, *a*, and the shortest diameter, *b*, were measured to calculate the degree of flattening, as shown in Equation 1.

Equation 1: $$\textit{Degree of Flattening} = [1 - (a/b)] \times 100\ \%$$

2.2.4 DNA Quantification

To verify that increased follicle size represented increased follicle cell number, DNA quantification was performed on follicles cultured in the orbiting test tubes in the presence and absence of FSH. At 72 hours of culture, DNA was extracted from eight follicles per treatment group and was quantified using the fluorescent dye, Hoechst 33258 (bisbenzimidazole; Sigma), and a microplate fluorescence reader (Perkin Elmer Life Sciences, Boston, MA) at 365 nm excitation and 450 nm emission wavelengths [67]. A range of dilutions of salmon testes DNA (Sigma) was used as a standard from which an average content of DNA per follicle (ng/follicle) was extrapolated.

2.2.5 Calcium Alginate Gel Encapsulation

Prior to being placed in culture, some rat and mouse follicles were encapsulated in calcium alginate gel beads in an effort to reduce shear stresses exerted on the follicle itself and to provide an environment in which spatial control of follicle-follicle interactions could be analyzed in the future. Once isolated from the ovary 20 to 30 follicles were transferred with glass pipettes to a solution of sodium alginate (1-2 % w/v; Sigma) in distilled water. The mixture of follicles in sodium alginate was slowly released through a 25 gauge needle as droplets falling into a beaker containing a stirred solution of $CaCl_2$ (0.1 M). The droplets immediately gelled to form beads. A stream of 0.2 μm filtered air was positioned at the tip of the needle to cut the mixture stream into small droplets to obtain beads with diameters between 250 μm to 500 μm. The sodium alginate and calcium chloride solutions were each syringe filtered through a 0.2 μm membrane and maintained at 37 ^{0}C throughout the entire process.

Beads containing follicles were then removed from the beaker using glass pipettes and immediately transferred to media in a well, tube, or rotating-wall vessel. The cultures were placed in the incubator for a period of 72 hours during which time follicle diameter and morphology were examined on a daily basis to determine the impact of encapsulation.

2.2.6 Statistical Analysis

Between 25 and 60 follicles were analyzed for each treatment group. Data points in figures represent mean follicle diameter and error bars represent the standard error of the mean. The level of statistical significance between mean values was determined by repeated measures analysis of variance (ANOVA) followed by Student Newman Keuls or Tukey post-hoc tests. Significance between points was accepted at the $p<0.05$ level.

2.3 RESULTS AND DISCUSSION

2.3.1 The Role of FSH on Growth of Preantral Follicles

Rat preantral follicles were cultured in the presence and absence of Follicle Stimulating Hormone (FSH) by the conventional culture system to evaluate the effect of FSH on growth and morphology over 72 hours. Conventionally cultured preantral follicles in the absence of FSH (CCS Control) had a slight (2.4 %) but statistically insignificant ($p>0.05$) increase in diameter (Figure 8). Follicles cultured in the presence of FSH (CCS+FSH) had a 20.5 % ($p<0.05$) increase in average diameter during the culture period.

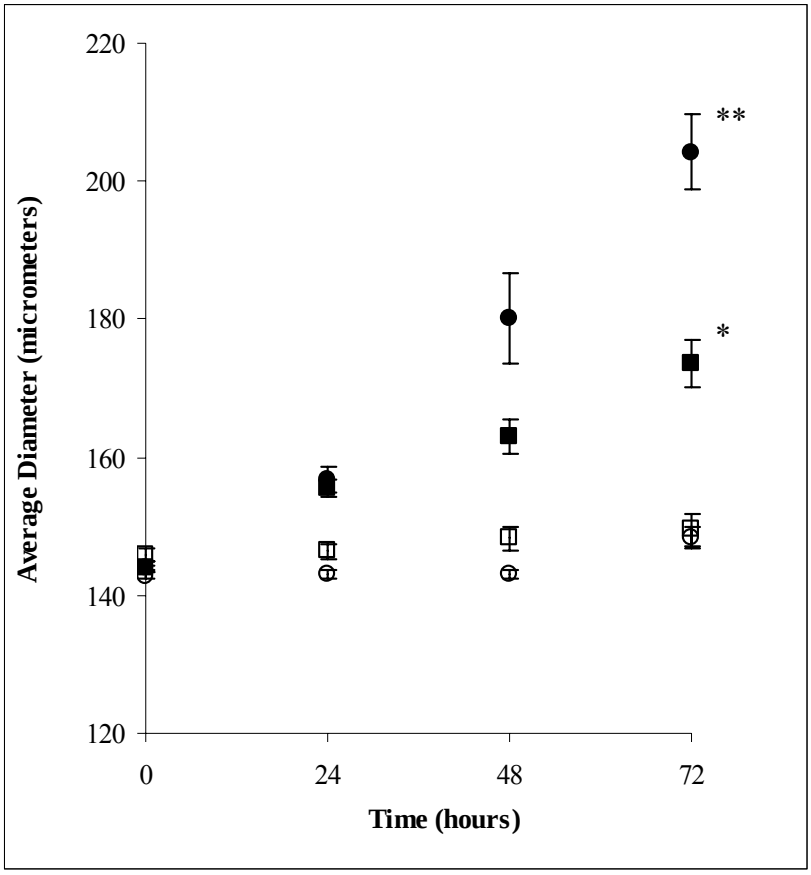


Figure 8: Rat Preantral Follicle Growth. Rat preantral follicles were cultured in the absence of FSH in the Conventional Culture System (CCS Control □) and in the Orbiting Test Tubes – Suspension Culture System (OSCS Control ○). Follicles were also cultured in the presence of FSH in the Conventional Culture System (CCS+FSH ■) and in the Orbiting Test Tubes – Suspension Culture System (OSCS+FSH ●). Follicle diameter was measured daily using an inverted microscope and approximately 30 follicles were analyzed per treatment group. Data points represent an average diameter ± SEM. One asterisk, ∗, represents a significant difference ($p < 0.05$) from both control groups, CCS Control and OSCS Control. Two asterisks, ∗∗, represents a significant difference ($p < 0.05$) also from the CCS+FSH group.

2.3.2 Follicular Flattening and Rupture in the Conventional Culture System

In these experiments, follicles were analyzed for flattening and rupture to determine if these changes are a significant occurrence in the conventional culture system (Figure 9). Approximately 53.5 % of the follicles cultured in the CCS+FSH group had an average diameter increase of 25 % ($p<0.05$) but flattened by a degree ranging from 5.1 % to 26 % (average of 14

%). Follicle rupture only occurred in CCS+FSH cultures, between 48 to 72 hours in culture, and comprised of 16.3 % of the group. At the end of the 72 hour culture period, only 30.2 % of the follicles in the CCS+FSH group remained spherical. These follicles had a slower rate of growth than the other groups of follicles, with an average increase in diameter of 12.5 % ($p<0.05$). The high rate of disruption in follicle morphology in static culture leads to the conclusion that an alternative culture technique is needed to maintain ovarian follicles for long-term developmental studies.

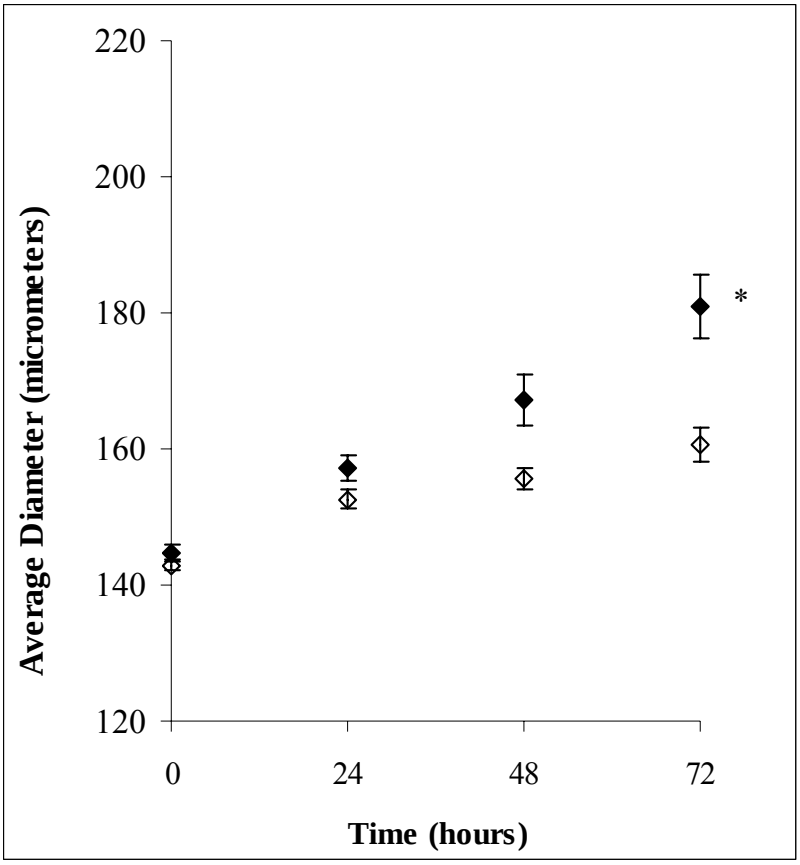


Figure 9: Rat Preantral Follicle Growth in the Conventional Culture System. Rat preantral follicles were cultured in the presence of FSH in the Conventional Culture System. The diameter growth was measured for follicles that appeared to flatten (CCS+FSH flattened ◆) and follicles that remained spherical (CCS+FSH spherical ◇). Follicle diameter was measured daily using an inverted microscope and approximately 30 follicles were analyzed per treatment group. Data points represent an average diameter ± SEM. An asterisk, ∗, represents a significant difference ($p < 0.05$) from the CCS+FSH spherical group.

2.3.3 Follicular Flattening and Rupture in Suspension Culture Systems

2.3.3.1 Orbiting Test Tube – Suspension Culture System (OSCS)

The mechanism of the flattening and rupturing is not known but is presumably a combination of biologic signals that result from the artificial culture environment of the conventional wells. Our goal is to understand which features of the artificial environment contribute significantly to the observed morphologic changes. Static culture wells are likely to experience poor nutrient delivery and we therefore explored the impact of gentle agitation on the growth rate and morphology of cultured follicles in a slowly orbiting test tube system.

As shown in Figure 8, rat preantral follicles cultured with FSH in the slowly orbiting test tubes (OSCS+FSH) grew to approximately the same size as the follicles cultured in the static wells (CCS+FSH) during the first 24 hours. However, after the 24 hours, the follicles cultured in the tubes had an increased growth rate ($p<0.05$). Overall, the follicles from this group had a 42 % increase in average diameter growth by the end of the 72 hour culture, double that obtained in the conventional culture system. Importantly, neither flattening nor rupture of follicles cultured in tubes was observed (Figure 13).

To determine if an increase in diameter corresponded to an increase in cell number, the DNA content per follicle was compared for groups of rat follicles cultured in the tubes for 72 hours in the absence or presence of FSH (Table 3). The follicles cultured in the presence of FSH (OSCS+FSH) contained approximately double the amount of DNA than the follicles cultured in the absence of FSH (OSCS Control). The average diameter of the OSCS+FSH group was also approximately double the average diameter of the OSCS Control group, indicating that an

increase in diameter indeed corresponds to an increase in cell number for follicles cultured in the orbiting test tubes in the presence of FSH.

Table 3: DNA Quantification. The percent growth, diameter, and amount of DNA, was measured for eight follicles cultured in the absence of FSH (OSCS Control) and eight follicles cultured in the presence of FSH (OSCS+FSH), both in the orbiting test tubes. The values are recorded as averages ± SEM. An asterisk, *, represents a significant difference ($p<0.05$) from the OSCS Control group.

	Percent Growth (%)	**Diameter (μm)**	**Amount of DNA (ng)**
OSCS Control	1.7 ± 1.0	144.1 ± 1.4	40.4 ± 8.1
OSCS+FSH	31.7 ± 2.9*	186.6 ± 4.0*	80.1 ± 9.2*

2.3.3.2 Rotating-Wall Vessel – Suspension Culture System (RSCS)

Although culture in the orbiting test tubes increased the rate of growth, we hypothesized that more aggressive mixing of the media could be beneficial. Rotating-wall vessels have been used to culture complex tissues, and one might predict they would be useful for follicle culture. However damaging shear stress could be exerted on cells or tissue due to the drag force of the rotating media. The prediction that incubating follicles in a miniature rotating-wall vessel would affect their viability was tested experimentally. Out of fifty-five follicles cultured with FSH for 72 hours in the rotating-wall vessels 16.4 % did not grow and 74.5 % fragmented, but interestingly 9.1 % of the follicles experienced a rapid diameter increase of 29.3 % (Figure 10). These follicles had spherical morphology, but the darkened appearance of the granulosa layer suggests that the follicles may have experienced some damage.

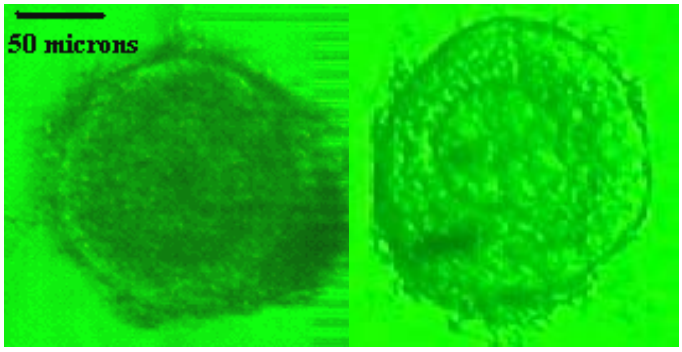


Figure 10: Rat Preantral Follicles Cultured in the Rotating-wall Vessels. *Left:* Follicle with darkened granulosa cells; *Right:* Follicle with no apparent damage.

2.3.4 The Impact of Microencapsulation on Follicle Growth and Morphology

In order to protect follicles from direct shear stress while maintaining the positive aspects of suspension culturing, we encapsulated rat, and for comparison, mouse follicles in alginate beads. A semipermeable hydrogel, like calcium alginate, allows substrates in the culture media with a molecular weight of less than 3×10^5 to easily diffuse through the gel [73]. Alginate is the most widely used polymer for cell encapsulation due to its biocompatibility, high porosity, and ability to form gel beads that are strong enough to withstand shear forces that typically exist in a bioreactor [73].

Our data show an average increase in diameter of 24.1 % for rat preantral follicles (Figure 11) and 29.3 % for mouse preantral follicles (Figure 12), encapsulated in calcium alginate beads. This is less than the increase obtained with non-encapsulated follicles in orbiting test tubes, but twice that of follicles in conventional wells. Unlike the non-encapsulated follicles however, neither flattening nor rupture occurred among the encapsulated follicles. They were more spherical and appeared similar to natural morphology, as shown in Figure 13. Therefore, it

is evident from our data that microencapsulating follicles in calcium alginate provides the follicle with support and protection from shear stress when cultured in the rotating-wall vessels.

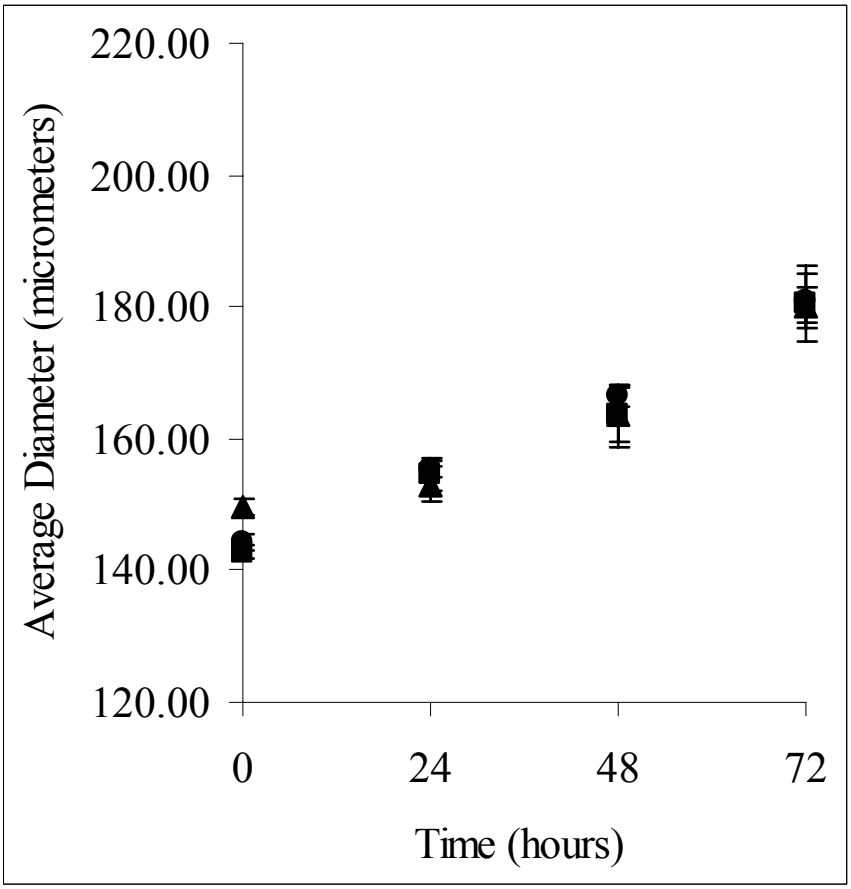


Figure 11: Growth of Encapsulated Rat Preantral Follicles. Rat preantral follicles were encapsulated and cultured in the presence of FSH in the Conventional Culture System (CCS+FSH ■), in the Orbiting Test Tubes – Suspension Culture System (OSCS+FSH ●), and in the Rotating-wall Vessels – Suspension Culture System (RSCS+FSH ▲). Follicle diameter was measured daily using an inverted microscope and approximately 20 follicles were analyzed per treatment group. Data points represent an average diameter ± SEM.

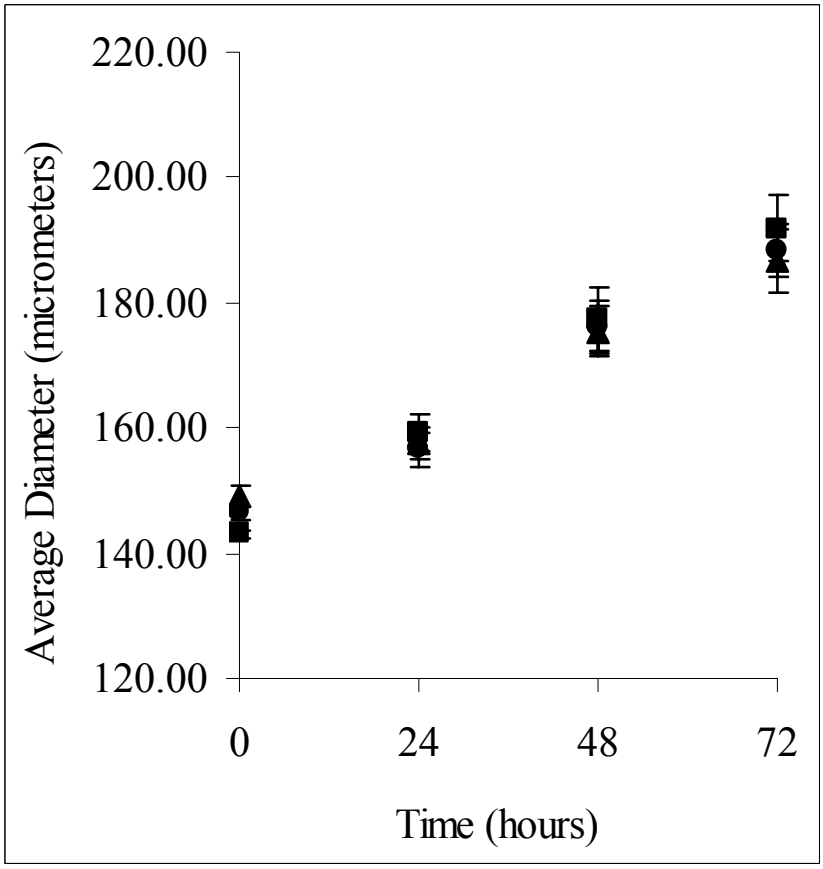


Figure 12: Growth of Encapsulated Mouse Preantral Follicles. Mouse preantral follicles were encapsulated and cultured in the presence of FSH in the Conventional Culture System (CCS+FSH ■), in the Orbiting Test Tubes – Suspension Culture System (OSCS+FSH ●), and in the Rotating-wall Vessels – Suspension Culture System (RSCS+FSH ▲). Follicle diameter was measured daily using an inverted microscope and approximately 20 follicles were analyzed per treatment group. Data points represent an average diameter ± SEM.

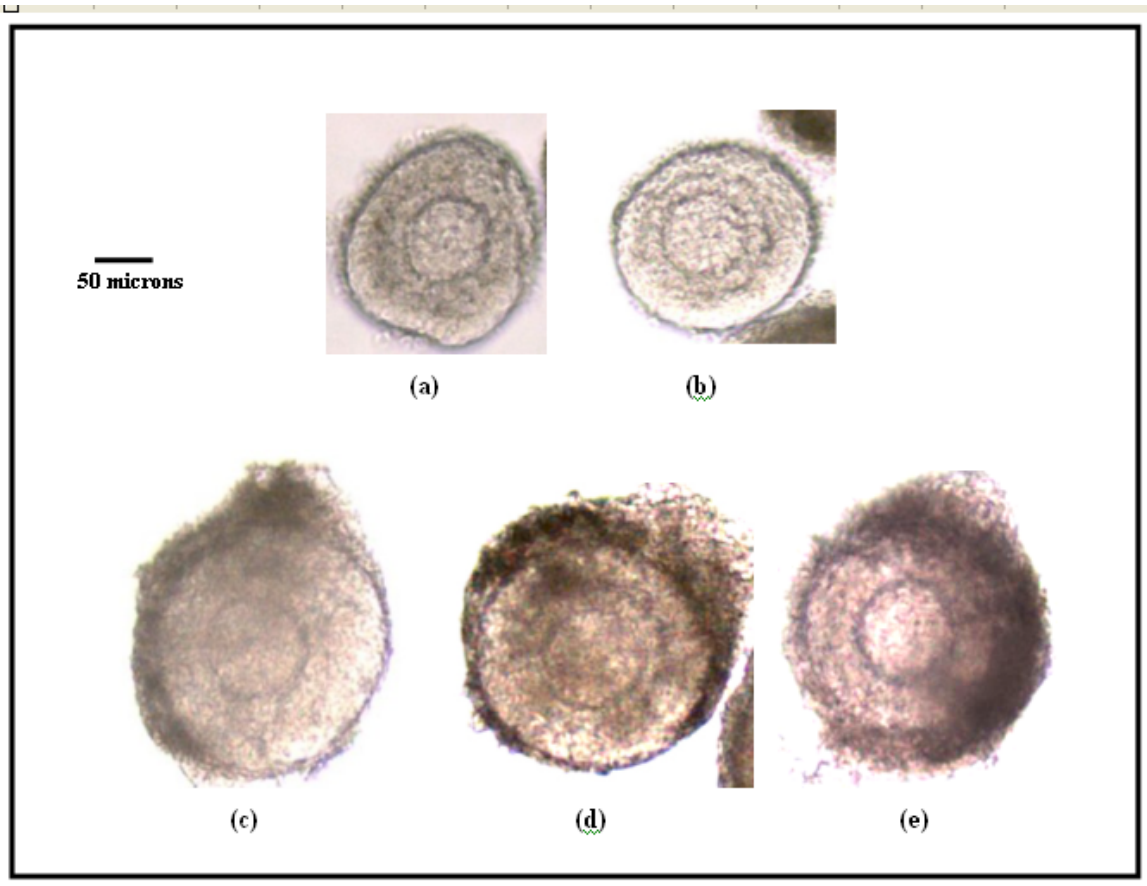


Figure 13: Rat Preantral Follicles. Rat preantral follicles were cultured for 72 hours in the absence of FSH in **(a)** the conventional culture wells, and in **(b)** the orbiting test tubes. Rat preantral follicles were also cultured for 72 hours in the presence of FSH **(c)** in the conventional culture wells, **(d)** in the orbiting test tubes, and calcium alginate encapsulated in **(e)** the rotating-wall vessels.

2.4 CONCLUSIONS

We investigated features of conventional culture that may contribute to changes in follicular morphology. This study shows that the majority of rat follicles cultured in the conventional system loose anatomical integrity by 72 hours of culture. Although prolonged static culture of intact preantral follicles has been successful in mice, [1, 67] similar success with species that have larger follicles has not been reported. For this reason, the mouse model has become a

standard for in-vitro maturation of ovarian follicles. By comparing the growth and morphology of mouse follicles grown in different culture systems, the advantages and disadvantages of a particular culture technique can more easily be determined. The rate of follicle development and ultimate size varies between species [1] and could be a reason why conventional culture is not as successful for larger follicles from species, such as rat, cat, sheep, and human.

This study is the first to apply a new environment for the culture of rat preantral ovarian follicles. We demonstrated that suspension culture in combination with hydrogel microencapsulation maintains follicular growth and morphology more effectively than conventional culture in tissue culture wells. In contrast to static culture, follicles are uniformly supported during culture in a rotating-wall vessel, unable to adhere to a surface, and are protected from shear stress when embedded in calcium alginate microcapsules. The cultures in this study, however, were short term and therefore studies of long term follicle culture in a rotating-wall vessel must be performed to optimize the culture system for follicle maturation. Early follicles are avascular but by the time a follicle reaches the antral stage *in vivo* the theca layer has become vascularized with capillaries that continue to expand during follicle growth. It is therefore likely that as a follicle grows the oxygen tension and vessel-wall rotation rate may need to be adjusted. Further study is needed to determine if this technique will be applicable to prolonged culture of ovarian follicles and which conditions are most appropriate for optimal follicle growth.

This investigation focused on alterations of follicle architecture that occur in the conventional culture system. We have developed a method that maintains preantral follicular morphology during culture. Follicular maturation is a complex, prolonged, and dynamic process that is not yet completely understood. More investigations of cultured microencapulated follicles

in the rotating-wall vessel are necessary to further develop this system to support the entire maturation process.

3.0 CALCIUM ALGINATE MICROENCAPSULATION OF OVARIAN FOLLICLES IMPACTS FSH DELIVERY AND FOLLICLE MORPHOLOGY

3.1 INTRODUCTION

The culture of intact ovarian follicles is useful for the study of the regulation of folliculogenesis, but may also provide an alternative to ovarian transplant for the preservation of fertility [74]. Classically, ovarian follicle culture has been performed in culture dishes, on a flat surface. Although ovarian follicles from the mouse can readily be grown on culture plates [52, 75], the maturation of follicles from larger mammals has proven to be much more difficult [74]. One cause of difficulty may be that larger follicles have different structural needs. Mouse follicles can ovulate at 400 μm in diameter [76], whereas rat preovulatory follicles are often greater than 800 μm in diameter [77]. Though this represents only a doubling of diameter, the *volume* of the rat preovulatory follicle is at least 8 times that of the mouse. The application of standard tissue engineering principles to the growth of ovarian follicles *in vitro* may provide additional tools necessary to overcome the structural challenges presented by follicles from larger mammals and make significant advances in this field.

It has been shown that suspension culture both enhances cell proliferation [78] and prevents follicle flattening and maintains three-dimensional follicle architecture better than culture on flat plates [79]. Though mouse follicles grown in a hanging drop system grew very

well compared to follicles grown on flat plates, the follicle diameter was still well under 500 after 6 days of culture [78]. Rat follicles often rupture at 200 to 250 μm on flat plates. However in suspension culture follicles routinely maintain intact survival to about 400 μm. Past this size, basement membrane integrity is lost and the three-dimensional structure of the follicle is altered. One tissue engineering approach to this problem is to encapsulate the follicles. Microencapsulation has been used to provide structural support for a variety of tissues such as pancreatic islets [80] and thyroid follicles [81].

Alginate is one of the most commonly applied biomaterials for microencapsulation due to its biocompatibility, high affinity to water, and ability to form gels under mild conditions when in the presence of calcium ions [73, 82-84]. Alginate is comprised of chains of alternating blocks of mannuronic acid, which contributes the elastic property of the gel; and guluronic acid, which contributes mechanical strength, stability, porosity, and gel forming properties [71, 85]. Alginates are extracted from all species of brown algae and contain differing compositions of mannuronic acid/mannuronic acid, mannuronic acid/glucoronic acid, and glucoronic acid/glucoronic acid blocks offering a variation in strength and stability. Alginate gel beads are reported to have a high porosity range and only limit the diffusion of large proteins [72]. It has been reported that substrates of molecular weight less than 2×10^4, such as glucose, L-tryptophan (MW = 204), and α-lactoalbumin (MW = 1.54×10^4), are able to diffuse freely into and from calcium alginate beads at approximately the same diffusion rate as in water, while larger proteins, such as albumin (MW = 6.9×10^4), could not diffuse freely into the calcium alginate beads [86]. Although there is resistance for larger proteins (MW > 2×10^4) diffusing into these beads, diffusion from the bead into a surrounding solution devoid of the substrate is not hindered until the molecular weight of the substrate approaches 3×10^5 [73]. FSH has a

molecular weight of 3×10^4 [87]. Thus, FSH is in the size range where hindrance may play a role in FSH availability to follicles encapsulated in calcium alginate beads.

In this study, the role of a calcium alginate scaffold on follicle growth, survival, and morphology in suspension culture was explored. Our previous studies suggested that alginate encapsulation slowed follicle growth [79]. Therefore, in these studies we have determined the growth rates of follicles in response to different delivery methods of FSH to encapsulated follicles. As a result, we have established the ability of calcium alginate to support three-dimensional follicular growth of intact follicles, but discovered that encapsulation may limit follicular access to FSH unless it is included in the alginate bead itself.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Ovarian Dissection

All animal experiments were performed in accordance with National Institutes of Health guidelines and with institutional approval. Sprague-Dawley rats were obtained from Hilltop Lab Animals (Pittsburgh, PA) and housed under standard conditions. The animals were sacrificed by CO_2 exposure and cervical dislocation. Ovaries were carefully dissected and placed immediately in warmed culture medium, consisting of Leibovitz L-15 Medium (Gibco BRL) with 1% bovine serum albumin (Sigma). The follicles were then mechanically dissected from the ovary as previously described [69]. All follicles used in the experiments were measured in two dimensions, using an inverted microscope fitted with an ocular micrometer. Only intact follicles

that were between 150 and 160 microns in diameter were used in culture. There was no statistical difference in the starting follicle diameter of any of the groups.

3.2.2 Calcium Alginate Microencapsulation

After dissection, 20 to 30 follicles per group were transferred with glass pipettes to a solution of sodium alginate (1% w/v; Sigma) in distilled water. (For the groups that required FSH in the bead, follicles were transferred to a solution of sodium alginate containing recombinant Follicle Stimulating Hormone, rFSH, (Serono Laboratories, Geneva) at a concentration of 1 iu/mL.) The mixture of follicles in sodium alginate was slowly released through a 25-gauge needle as droplets falling into a beaker containing a stirred solution of $CaCl_2$ (0.1 M). The droplets immediately gelled to form beads. A stream of 0.2 μm-filtered air was positioned at the tip of the needle to cut the mixture stream into small droplets to obtain beads with diameters between 250 μm to 400 μm. Beads containing individual follicles (Figure 14) were then removed from the beaker using glass pipettes and immediately transferred to media in 12 × 75 mm polypropylene culture tubes. Follicles were cultured one per tube.

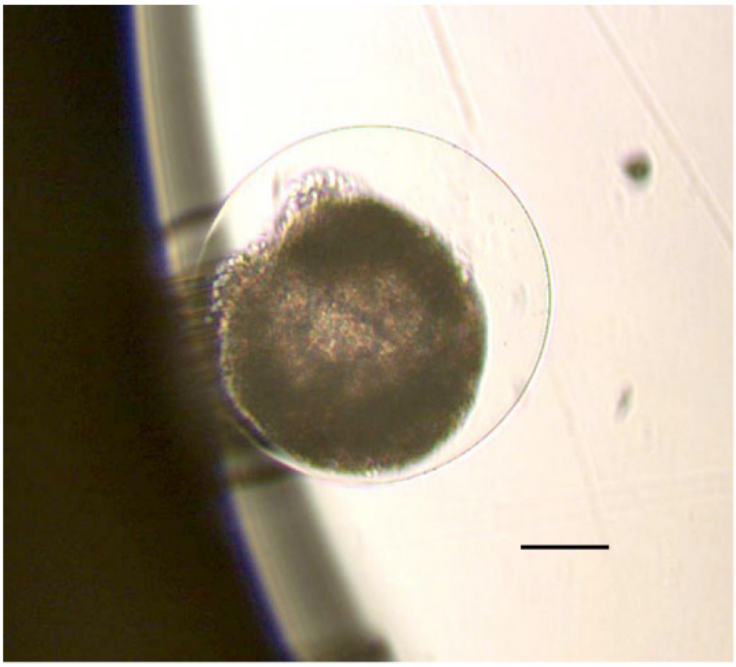


Figure 14: Ovarian Follicle Encapsulated in a Calcium Alginate Bead. Scale bar represents 50 μm.

3.2.3 Follicle Culture

Culture media consisted of α-Minimal Essential Medium (Gibco BRL, Invitrogen Corporation, Grand Island, NY) with additives of 8-bromo-cGMP (5 mM), ITS+ (1 % solution of insulin, 10 mg/L; transferrin, 5.5 mg/L; linoleic acid, 4.7 mg/L; selenium, 5 mg/L), Pen/Strep (1 %, penicillin 100 U/ml, streptomycin 100 μg/ml), all from Sigma Chemical Co. (St. Louis, MO), and recombinant Follicle Stimulating Hormone, rFSH, (1 iu/ml; Serono Laboratories, Geneva). In the basal or negative control group, FSH was not included. Culture media was placed into 12 × 75 mm polypropylene culture test tubes (500 μl/tube) and cultured in 5 % CO_2 and 37°C humidified incubator.

Suspension was attained by placing the 12 × 75 mm polypropylene culture tubes in a circular rotator plate (Glas-Col, Terre Haute, IN), having a diameter of 30.5 cm, which was

rotated around its horizontal axis at rate between 8–15 rpm. Therefore, as the plate rotates, the tubes slowly orbit the axis of the plate and the follicle is maintained is fluid suspension. After 72 hours in culture, follicle diameter was measured once again and follicles were collected for DNA quantification and histological analysis. For survival analysis, follicles were observed daily for basement membrane integrity and signs of atresia. Atresia is readily apparent as darkening of the follicle appearance under the dissecting microscope [64, 79]. Survival analysis cultures were continued for 7 days.

3.2.4 DNA Quantification

To verify that increased follicle size represented increased follicle cell number, DNA quantification was performed on the cultured follicles as previously described [79]. At 72 hours of culture, the follicles were released from the beads by allowing the capsules to dissolve in sterile PBS (pH 7.4). Follicles (n = 5 for each group) were then trypsinized and DNA was extracted from each individual follicle and quantified by using the fluorescent dye, Hoechst 33258 (bisbenzimidazole; Sigma), and a microplate fluorescence reader (Perkin Elmer Life Sciences, Boston, MA) at 365 nm excitation and 450 nm emission wavelengths [88]. A range of known dilutions of salmon testes DNA (Sigma) was used to plot a standard curve from which follicle DNA content was extrapolated as previously described [79]. This experiment was repeated twice with the same result.

3.2.5 Histology and Immunohistochemistry

To confirm that cultured follicles encapsulated in calcium alginate retain normal anatomic relationships between the cell types within a follicle, immunohistochemistry for connexin 43 was performed on follicles that were cultured in calcium alginate beads with and without FSH. Some additional sections were also stained with hematoxylin and eosin. At 72 hours of culture, the follicles were released from the beads by allowing the capsules to dissolve in sterile PBS (pH 7.4). Follicles were embedded in OCT for fluorescent immunostaining.

Frozen sections were cut at 5 μm and air-dried overnight at room temperature. Sections from at least 10 follicles per group were evaluated for connexin staining pattern. After a 10 minute acetone fixation, slides were again allowed to air dry, and then were rehydrated in PBS for 10 minutes. Immunohistochemistry was performed using the M.O.M. immunodetection system (Vector Laboratories, Burlingame, Calif.). All reagents were prepared according to kit directions and all steps were carried out at room temperature. Slides were incubated with blocking reagent for 1 hour. Mouse anticonnexin (Chemicon International, Temecula, CA) was prepared in diluent to a final concentration of 0.1 μg/mL and applied to the sections for 1 hour in a humidified chamber. For negative control sections, mouse IgG was substituted for the anti-connexin antibody. After a PBS wash, sections were incubated with biotinylated anti-mouse IgG for 10 minutes. Slides were again washed for 10 minutes in PBS, and then incubated with fluorescein avidin DCS (1:62.5 in PBS) for 5 minutes. After a final 10 minute PBS wash, slides were mounted with Vectashield mounting medium (Vector Laboratories) and digitally imaged using a Leica CMR fluorescence microscope.

3.2.6 Statistical Analysis

Statistical analysis for DNA quantification and follicle diameters was performed by analysis of variance (ANOVA) with post hoc testing. Survival data was analyzed with the Kaplan Meier program contained in the Sigma Stat 3.0 software package (Chicago, Il). Probability values less than 0.05 were used to determine significance.

3.3 RESULTS

3.3.1 Effect of calcium alginate encapsulation and FSH treatments on follicle diameter

Average follicle diameters at the beginning and end of the 72-hour culture period are depicted in Figure 15. To determine the effects of alginate alone, follicles were encapsulated in calcium alginate, but grown in the complete absence of FSH as a negative control. A positive control comparison group consisted of follicles grown in the standard fashion and treated with FSH, but without encapsulation [79]. As expected, the negative control group of follicles encapsulated and grown in the absence of FSH, experienced minimal growth (diameter increase; 6.3%). The positive control group of unencapsulated follicles with FSH in the medium experienced a 35.4% increase in diameter, consistent with our previous studies [79]. When encapsulated follicles were grown in medium containing FSH, a 19.9% increase in follicle diameter over the 72-hour culture period was observed ($p < 0.05$ relative to the negative control). When FSH was included in the calcium alginate capsule but not the medium, a 13.5% increase in follicle diameter was observed. This was different from the negative control ($p < 0.05$), but not the previously described FSH in

medium group ($p > 0.05$). However, when follicles were cultured with FSH included in both the medium and the bead, a 32.6% increase in follicle size was observed.

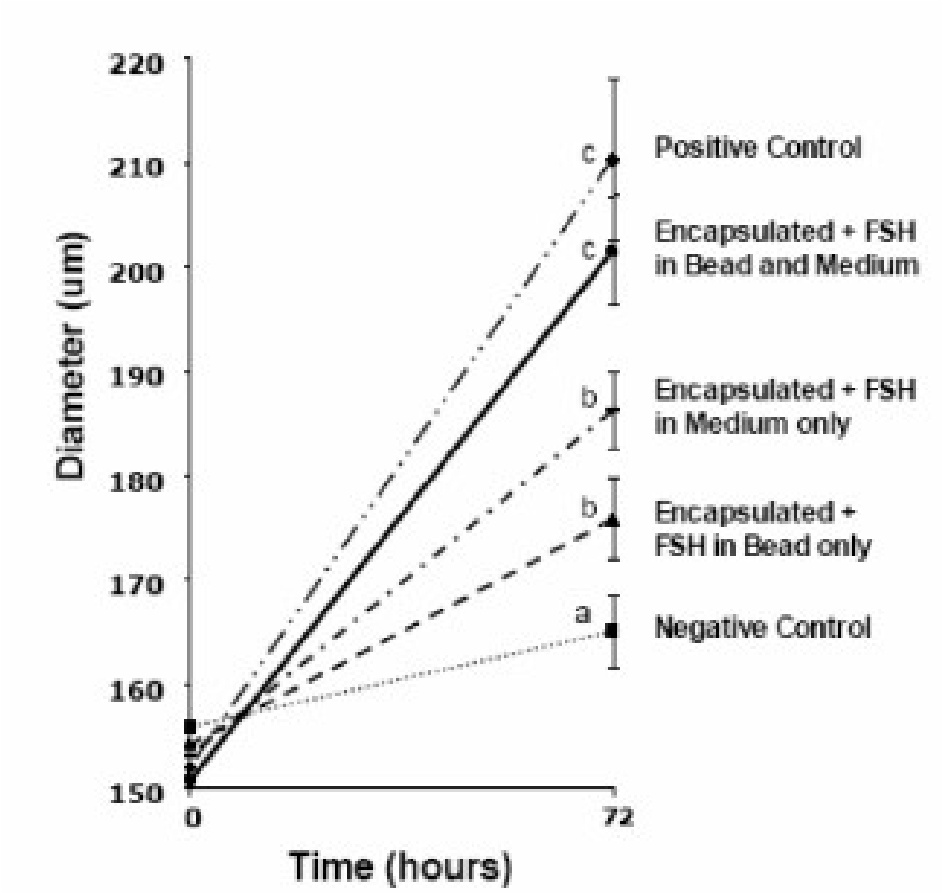


Figure 15: Diameter of follicles cultured under different conditions over 72 h. Each line represents measured follicle diameter before and after 72 h of culture (n = 20 – 30 follicles per group). Data points are average diameter with standard error bars included for each treatment. Treatment conditions are listed to the right of the data point representing average diameter at 72 hours for each group. a, b, c represent statistically different groups ($p < 0.05$).

The size of positive control follicles and encapsulated follicles with FSH placed in the medium and the bead were significantly different than the other three treatment groups (Figure 14, $p < 0.05$). Furthermore, there was no significant difference between the growth of unencapsulated FSH-treated cultures and the encapsulated follicles with FSH present in both the bead and the medium (positive control, 54.3 ± 10.1 μm; FSH in bead and media, 49.2 ± 6.8 μm; $p > 0.05$).

3.3.2 Survival Analysis

The effect of encapsulation on follicle survival was evaluated by Kaplan Meier analysis of daily observation of cultured follicles. Survival plots for unencapsulated and encapsulated FSH-treated follicles are shown in Figure 16. Mean intact survival time for encapsulated follicles, 6 days, was greater than the mean intact survival time for unencapsulated follicles, 4.5 days ($p < 0.05$).

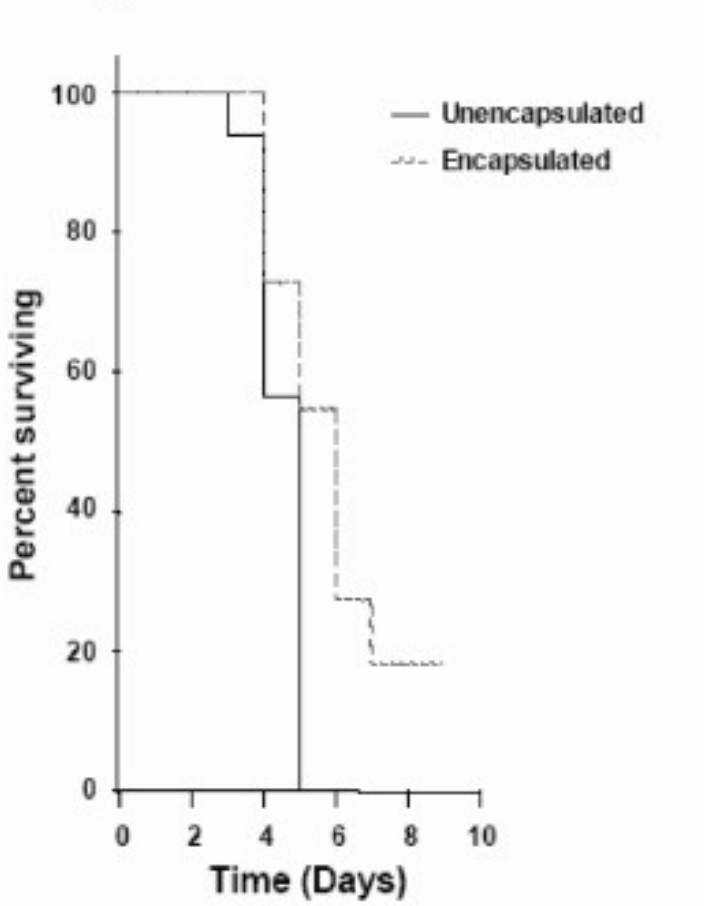


Figure 16: Kaplan Meier Survival Plot. Mean survival time for unencapsulated follicles is 4.5 days while mean survival time for encapsulated follicles is 6 days ($p < 0.05$).

3.3.3 DNA Quantification Confirms Follicle Growth

DNA content of individual follicles was consistent with measured follicle size. The unencapsulated follicles in FSH supplemented media (Positive Control) contained 50.7 ± 19.9 ng of DNA after 72 hours of culture. The encapsulated follicles with FSH present in the media and the bead (Experimental Group) contained 53.9 ± 15.9 ng of DNA, not significantly different from the positive control. Both FSH-treated groups contained significantly greater amounts of DNA than the encapsulated follicles cultured in the absence of FSH (Negative Control) which had only 27.8 ± 11.7 ng of DNA ($p < 0.05$).

3.3.4 Connexin 43 Expression in Cultured Follicles

As seen in Figure 17A, connexin expression (green stain) is very low in follicles in the absence of FSH. In the presence of FSH, connexin expression is readily apparent in the unencapsulated follicle (Figure 17C). However when the follicle is encapsulated and treated with optimal FSH (both in the alginate and in the medium), excellent expression of connexin 43 is seen throughout the cross section of the follicle (Figure 17E). This pattern is very similar to the follicle grown *in vivo* (Figure 17G). The negative control pictured (Figure 17H) is a serial section from the same follicle from figure 17E and demonstrates that there is minimal background staining in this system. Follicle sections stained with H&E are adjacent to the corresponding connexin photograph. In the absence of FSH (Figure 17B), the follicle remains intact but the granulosa cell layer is poorly organized and contains numerous pyknotic appearing cells. Figure 17D is of an FSH treated follicle that was not encapsulated. There is a thickened appearance of the outer granulosa cells though the cells around the oocyte appear fairly normal. This is consistent with

the area that stains more heavily for connexin. Figure 17F depicts a typical follicle grown in the bead with optimal FSH. There is good morphology with a fairly uniform granulosa layer and a healthy appearing oocyte.

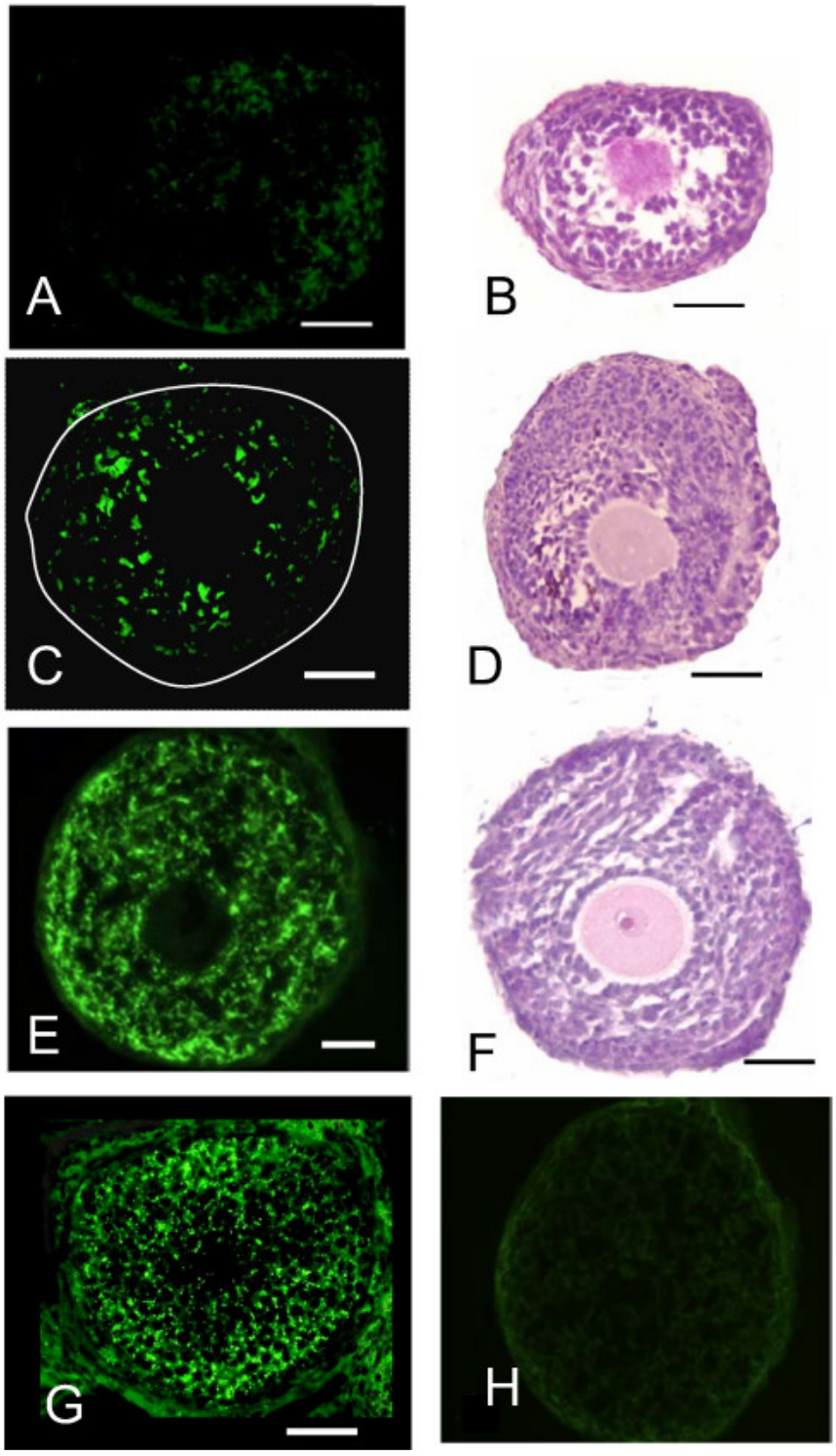


Figure 17: Connexin 43 expression and histology of representative cultured follicles. (A and B) Encapsulated follicle in the absence of FSH, (C and D) Unencapsulated follicle grown in FSH supplemented media, (E and F) Encapsulated follicle with FSH present in both the media and the bead, (G) Connexin staining in preantral follicle of an intact ovary section. (H) Negative control for the immunohistochemical staining, serial section from the same follicle as in C. A, C, E, G, H, represent connexin immunohistochemistry. B, D, and F are H&E stained follicle sections. In panel C, white line is location of basement membrane. Scale bars represents 50 μm in length.

3.4 DISCUSSION

In this study, we have demonstrated that calcium alginate encapsulation can act as a scaffold to support three-dimensional relationships between the cells of a follicle in suspension culture. However we have also demonstrated that calcium alginate encapsulation can impede access of the follicle to FSH in the medium.

We have previously shown that FSH is a growth factor for preantral follicles in culture [70]. Other investigators have shown that the dosing and timing of follicle exposure to FSH has threshold limits for continued growth and survival of mouse follicles [88]. Our previous work has demonstrated that encapsulation slowed the rate of follicle growth [79]. There were two hypotheses considered to explain the decreased growth response to FSH. Either FSH diffusion into the alginate bead was significantly limited, or the bead itself physically hindered the growth of the follicle.

With FSH present only in the bead, a concentration gradient is created between the bead and the media. Naturally this gradient would drive the flux of FSH out of the calcium alginate capsule. For this case, because the molecular weight is less than 3×10^5, the FSH will freely diffuse out of the bead until equilibrium is reached [86]. For a substrate with the molecular weight of FSH, equilibrium would be reached within 3 to 5 hours [73], resulting in a much lower concentration of FSH within the bead for the bulk of the 72 hour culture. As a result, follicular growth response was greatly diminished compared to the positive control (Figure 15).

Similarly, by adding FSH to only the media, a concentration gradient is established in the opposite direction, driving the flux of FSH from the media into the bead. However, due to the molecular weight of the hormone being greater than 2×10^4, the diffusion into the capsule will

be impeded [73]. The question is then, will the decreased diffusion rate into the calcium alginate bead be significant enough to cause the observed reduction in follicular growth (Figure 15).

To answer this, equal concentrations of FSH were placed in both the bead and the culture media. By eliminating the concentration gradients, FSH was not driven from the bead, leaving the concentration of FSH within the bead comparable to that present for the positive control. Therefore, because the addition of FSH to the alginate bead restored the follicular growth rate to that of the positive control, the likely cause of the diminution of follicle growth in our earlier studies [79] is a limitation of follicle access to FSH by the encapsulation process.

In order to confirm appropriate cell-cell interactions within the follicle, we next evaluated the expression of the gap junction protein connexin 43 in cultured follicles. Connexin 43 plays a key role in follicle development by promoting communication between granulosa cells via the exchange of small ions and molecules [89, 90]. Connexin 43 deficient mice have severely impaired follicle development beyond the primary stage [91]. The expression and localization of connexin 43 is also highly FSH dependant [89]. The evaluation of connexin in our cultured follicles provides both a structural and a hormonal assessment of the effect of alginate on follicle development and FSH-stimulated differentiation.

In this study, it was found that the patterned expression of connexin 43 is not affected by calcium alginate encapsulation when the FSH is included in both the bead and the medium (Figure 17). Encapsulated preantral follicles cultured with FSH, maintain a well developed theca, a basement membrane, a normally configured granulosa compartment and a healthy appearing egg with a zona pellucida (Figure 17E and 17F). Interestingly, it was observed that the positive control lacked the punctuate staining at its periphery (Figure 17C), while the encapsulated follicle demonstrated consistent punctuate staining throughout the entire granulosa layer (Figure

17E) similar to *in vivo* preantral follicles (Figure 16G). This may suggest that encapsulating the follicle might protect it from possible deleterious shearing forces in the suspension culture system, since shear stress has been shown to affect connexin expression [92]. Further studies will be needed to more fully evaluate this phenomenon.

Although a variety of gels and scaffolds exist, calcium alginate has some desired advantages. Unlike collagen based gels [93, 94], the follicle can be easily viewed through the calcium alginate capsule, allowing for daily observations to be made. Alginate is easily washed away with PBS, unlike collagen. The alginate also does not seem to interact with the follicles unless ECM or its components are included in the gel [95]. Matrigel has also been used as an imbedding medium for follicles. However matrigel is like serum in that it is derived from undefined biological sources and can have considerable content variation from lot to lot. In contrast, calcium alginate is a well-defined gel and fits with the goal of establishing a completely defined culture system that can be stably reproduced and still meet all the nutritional and structural needs of the developing follicle.

3.5 CONCLUSIONS

These combined findings have demonstrated that microencapsulation can improve the support of three dimensional growth of preantral follicles; but requires the inclusion of FSH in the scaffold. Further work is necessary to determine if intact follicles can be maintained for longer periods in culture with this scaffolding approach. However, care must be taken to determine if the scaffolding material itself alters the microfollicular environment. Though follicle development is a prolonged and complex process, careful application of tissue engineering principles may

facilitate the eventual development of a consistent, standardized *in vitro* process for follicle growth of large mammals that will be reliable enough for gamete production.

4.0 DYNAMIC OXYGEN ENHANCES OOCYTE MATURATION IN LONG TERM FOLLICLE CULTURE

4.1 INTRODUCTION

Primordial follicles have a greater likelihood of surviving this cryopreservation due to their smaller size, slow metabolism, and the undifferentiated state of the cells that comprise them [4]. Although primordial follicles derived from mice can be grown to maturity using static culture techniques under atmospheric oxygen conditions [5-7], to date there has been no successful complete *in vitro* follicle development from larger species, such as rats, pigs and humans [8]. In other words, intact rat, primate, or human preantral follicles have not yet been successfully cultured to maturation *in vitro*. Successful development of a technology in which preserved ovarian tissues can be utilized to restore fertility, would be a major development in reproductive science.

Over the past several years, several groups have worked to develop a defined culture system for preantral follicular development. This work has identified a number of factors important for follicular growth *in vitro*. It has been shown both *in vivo* and *in vitro*, that preantral follicles respond to FSH treatment with increased cell division, growth and differentiation [70]. Other factors that promote growth or differentiation of rat preantral follicles in short term culture have been identified (Mullerian Inhibitory Substance [96], Keratinocyte

Growth Factor [97], Growth and Differentiation factor 9 [98] and activin [96]). It has also been determined that TGF-β induces apoptosis [96], whereas cGMP analogs inhibit apoptosis in preantral follicles [70]. While these studies have added to our knowledge of early follicle development and greatly improved the media used for *in vitro* culture of isolated follicles, a number of obstacles still remain before follicles can be supported in the proper environment long enough to result in normal oocyte maturation.

We have recently performed studies to determine why rat follicles fail to progress beyond the preantral follicle stage *in vitro*. Our preliminary studies [79] demonstrated that under conventional conditions, rat follicles frequently undergo flattening and rupture with loss of anatomic integrity that is important to the normal egg maturation process. When follicles were cultured in suspension culture systems, consisting of orbiting test tubes and rotating-wall vessels, the follicles did not rupture and exhibited more robust growth. Therefore, we believe that advances in bioreactor technologies hold great promise toward the development of a system that can support the complex needs of larger follicles that are required for proper development. In the short term, the rat model will allow for more complex studies of follicle development *in vitro* that are unnecessary for the maturation of the smaller mouse follicles. In the long term it may represent the hope for young cancer patients to retain fertility. More generally, the problems associated with nurturing the development of larger follicles mirror many of the unmet challenges in tissue engineering as a whole.

Follicles have traditionally been grown in standard incubators with atmospheric oxygen concentrations. However, primordial and primary follicles exist in the avascular cortex of the ovary. Throughout this region, blood vessels are not observed in close proximity to these follicles [11]. The oxygen available to these small follicles would have to diffuse from the

peritoneal cavity or nearby large follicles that do have a blood supply. The partial pressure of oxygen in the peritoneal cavity has been measured at 40 mmHg, a far cry from the ~140 mmHg obtained in media exposed to normal incubator conditions [99]. Furthermore, the partial pressure of oxygen is expected to be even further reduced by crossing the ovarian capsule. The oxygen gradient across the walls of microcapillaries is steep, reducing the intra-capillary levels by half [100]. The Po_2 across the subrenal capsule drops from the peritoneal level of 40 mmHg to 14-19 mmHg [99]. In contrast, an abundance of blood vessels are found in the region of the ovary that contains secondary and antral follicles [11]. Differential regulation of follicle development in environments reflecting the *in vivo* Po_2 has never been investigated, though it is likely that *in vivo* follicles experience a transition from relative hypoxia in early development to the high volume of blood flow and oxygen delivery for pre-ovulatory follicles. The difficulty in achieving *in vitro* oocyte maturation may be due to the dysregulation of follicle development by exposure of early stage follicles to inappropriate oxygen concentrations.

In this study, we examine the effectiveness of a dynamic oxygen delivery protocol on oocyte viability and maturation. Ovarian follicles were cultured in suspension under two different oxygen conditions, a static 20% or a dynamic environment in which the oxygen is increased over the duration of the culture period. Along with an *in vivo* control group, the resulting oocytes are analyzed for survival, germinal vesicle breakdown, and polar body extrusion. Furthermore, additional oocytes derived from these three groups were placed into strontium chloride in order to determine the efficiency of parthenogenetic activation. As a result of the activation study, oocytes from the dynamic oxygen group underwent intracytoplasmic sperm injection (ICSI) to verify that they were competent to undergo fertilization.

4.2 MATERIALS AND METHODS

4.2.1 Animals and Ovarian Dissection

All animal experiments were performed in accordance with National Institutes of Health guidelines and with institutional approval. Sprague-Dawley rats were obtained from Hilltop Lab Animals (Pittsburgh, PA) and housed under standard conditions. The animals were sacrificed by CO_2 exposure and cervical dislocation. Ovaries were dissected and placed immediately in warmed culture medium, consisting of Leibovitz L-15 Medium (Gibco BRL). The follicles were then mechanically dissected from the ovary using a pair of syringes with 26 gauge needles. All follicles used in the experiments were measured in two dimensions, using an inverted microscope fitted with an ocular micrometer. Only intact follicles that were between 140 and 170 microns in diameter were used in culture.

4.2.2 Follicle Culture

Culture media consisted of α-Minimal Essential Medium (Gibco BRL, Invitrogen Corporation, Grand Island, NY) with additives of 8-bromo-cGMP (5 mM), ITS+ (1% solution of insulin, 10 mg/L; transferrin, 5.5 mg/L; linoleic acid, 4.7 mg/L; selenium, 5 mg/L), Pen/Strep (1%, penicillin 100 U/ml, streptomycin 100 μg/ml), all from Sigma Chemical Co. (St. Louis, MO), and recombinant Follicle Stimulating Hormone, rFSH (0.7 IU/mL), (Serono Laboratories, Geneva). Culture media was placed into 12 x 75 mm polypropylene culture test tubes (500 μl/tube) and cultured in 5% CO_2 and 37 ^{0}C humidified incubator. 200 μl of culture media was exchanged every 3 days.

Suspension culture was attained by placing the 6 ml culture tubes in a circular rotator plate (Glas-Col, Terre Haute, IN), having a diameter of 30.5 cm, which was rotated around its horizontal axis at rate between 8-15 rpm. Therefore, as the plate rotates, the tubes slowly orbit the axis of the plate.

4.2.3 Oxygen Protocols

The incubators used for this study were both CO_2 and O_2 controlled. For the ambient oxygen group, the oxygen tension was cultured in the traditional static oxygen tension of 20% (Figure 18). For the preliminary low oxygen experiment, the oxygen tension was set at 2% (15.2 mmHg). For the dynamic oxygen group, the oxygen tension in the incubator was initially set at 4% (30.4 mmHg), a concentration more similar to what a preantral follicle would experience *in vivo* [99]. In order to mimic the *in vivo* transition from avascular to vascular oxygen levels, the oxygen tension was increased 1% every 24 hours until the end of the 7 day culture period with a final oxygen tension of 11% (83.6 mmHg), a level within the normal range for arterial oxygen (Figure 18).

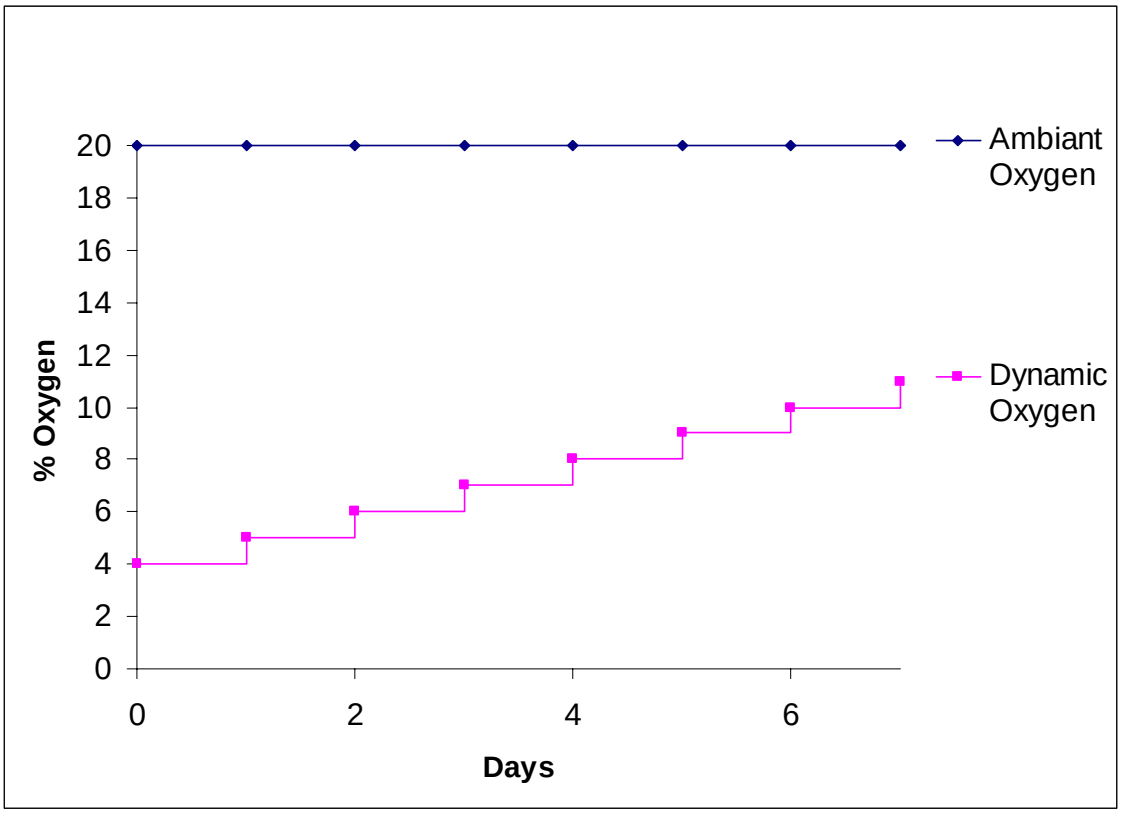


Figure 18: Dynamic Oxygen Delivery Protocol. The oxygen tension in the incubator was initially set at 4% (30.4 mmHg) and increased 1% every 24 hours until the end of the 7 day culture period with a final oxygen tension of 11% (83.6 mmHg).

4.2.4 Histology

The morphology of preantral follicles cultured at different oxygen tensions was analyzed by hemotoxylin and eosin staining. Preantral follicles were cultured in either 15% (115 mmHg) or 2% (15.2 mmHg) oxygen tension. After 7 days, the follicles were retrieved from culture and fixed in 4% paraformaldehyde. Follicles were then embedded in Optimal Cutting Temperature Compound for sectioning. Frozen sections were cut at 5 μm and stained with hematoxylin-eosin before being imaged using a Leica CMR microscope.

4.2.5 Ovulation Induction and Oocyte Maturation

Follicles were removed from the culture media and placed into media containing α̃-Minimal Essential Medium, Pen/Strep (1%, penicillin 100 U/ml, streptomycin 100 μg/ml), and hCG (1.0 IU/mL) for 12 hours. Oocytes were then removed from their surrounding cumulus and placed into IVF-30 media (Vitrolife). Oocytes were initially observed for viability. Oocytes with vacuoles in their cytoplasm or voids in their perivitelline space were considered non-viable. After 6 hours in the IVF-30 media, oocytes were examined for germinal vesicle breakdown and the presence of an extruded polar body. Oocyte diameter was measured from inside the zona pellucida using SPOT version 3.2.4 software (Diagnostic Instruments).

4.2.6 Superovulation Protocol

In order to compare the quality of the oocytes from both culture conditions with an *in vivo* control, immature Sprague-Dawley rats were stimulated to superovulate using pregnant mare's serum gonadotropin (PMSG) subcutaneous injections. Rats were injected with PMSG (15 IU) at 10 a.m. The rats were then injected with human chorionic gonadotropin (30 IU) 48 hours later. After 24 hours, the rats were sacrificed and the fallopian tubes dissected to retrieve the ovulated oocytes. The recovered oocytes were placed into the IVF media for 6 hours and examined for germinal vesicle breakdown and the presence of an extruded polar body.

4.2.7 Parthenogenetic Activation

Follicles were removed from the culture media and placed into media containing α̃-Minimal

Essential Medium, Pen/Strep (1%, penicillin 100 U/ml, streptomycin 100 μg/ml), and hCG (1.0 IU/mL) for 12 hours. Oocytes were then removed from their surrounding cumulus and placed into IVF-30 media (Vitrolife). In order to determine oocyte competency, oocytes from the dynamic oxygen group, the ambient oxygen group, and the *in vivo* control group were parthenogenically activated by using strontium chloride supplemented IVF-30 media. Only those oocytes that matured through germinal vesicle breakdown were put through the protocol. For mammalian oocytes, activation is triggered by intracellular calcium oscillations [101]. These calcium oscillations occur as intracellular calcium is released across the egg [102]. This process is necessary for the progression of the egg through the cell cycle [103]. Studies on mouse [104] and rat [105] oocytes have shown that strontium can provoke the calcium oscillations necessary for activation. However, in the case of strontium-induced parthenogenetic activation, there is no consensus on an optimum protocol regarding strontium concentration used and time spent in strontium that would result in the most efficient activation rates and the formation of the greatest number of blastocysts.

Strontium chloride (Sigma Chemical Company) was dissolved into IVF-30 medium at a concentration of 0.625 mM. After the 6-hour culture in IVF-30 media, oocytes from the dynamic oxygen group were placed into 50 μL drops of strontium supplemented IVF-30 media. They were incubated for 8 hours at 37^0C in 5% CO_2 in air. They were then washed in IVF media and cultured for 8 hours in IVF media alone. Finally, they underwent a second 8 hour culture in the strontium supplemented IVF media before being placed back into IVF media for 24 hours. Oocytes were then examined for the presence of 2 or more cells. For all incubation periods, the microdrops of IVF media were covered with mineral oil.

4.2.8 Oocyte DNA Staining

Activated oocytes were stained with Hoechst 33342 in order to confirm the presence of a nucleus in each cell. At the end of the activation protocol, oocytes that displayed 2 or more cells were placed into a 1 μg/ml working solution of the Hoechst 33342 for 10 minutes followed by two PBS washes, 5 minutes. Oocytes were then digitally imaged using a Leica DM IRB fluorescence microscope. Only those oocytes displaying positive staining in all cells were considered to be activated.

4.2.9 Intracytoplasmic Sperm Injection

ICSI was performed using a controlled volume microinjection system (Narishige) attached to a Leica DM IRB microscope. Spermatozoa were collected from the cauda epididymidis of a mature male Sprague-Dawley rat. The spermatozoa were suspended in a 10% polyvinylpyrrolidone solution (Irvine Scientific). Spermatozoa were then injected into the oocytes in a drop of HTF media supplemented with 10% v/v Synthetic Serum Substitute (Irvine Scientific). After injection, the oocytes were transferred into IVF-medium and incubated at 37^{o}C and 5% CO_2 in air. The oocytes used for injections were retrieved from follicles cultured in the dynamic oxygen condition and cultured for an additional 6 hours in the IVF media. Only those oocytes that matured through germinal vesicle breakdown were injected. The injected oocytes were observed over the next 24 hours for the presence of 2 pro nuclei and subsequent progression to a 2-cell embryo.

4.2.10 Statistical Analysis

The percentages of oocytes that were viable, went through germinal vesicle breakdown, and extruded the first polar body were compared between oxygen treatments using the Fisher exact probability test. Significance was accepted at $p < 0.05$.

4.3 RESULTS AND DISCUSSION

Since the overall goal of these studies is to develop a reliable method of attaining mature oocytes from *in vitro* cultured preantral ovarian follicles for the purpose of restoring fertility, the parameters used to compare the effects of the dynamic oxygen environment to those of the ambient control focused on oocyte viability and oocyte maturation. Oocyte maturation was evaluated by observing the progression of the cultured oocytes through a number of meiotic stages.

Oocytes that are still within a follicle are arrested in the prophase of the first meiotic division, the germinal vesicle stage (Figure 19A). Mature oocytes can spontaneously resume meiosis *in vitro* if they are retrieved from a follicle and separated from the surrounding cumulus cells (23). This induces a process known as germinal vesicle breakdown in which the nucleus dissolves in preparation for the completion of meiosis (Figure 19B). At the conclusion of meiosis I, the oocyte will extrude a polar body and enter meiosis II, at which point the oocyte will arrest in metaphase II until it is fertilized (Figure 19C). For the purposes of this study, oocytes were observed for germinal vesicle breakdown and the presence of a polar body in order

to evaluate the effectiveness of both the ambient oxygen and dynamic oxygen groups at producing mature oocytes.

Figure 20 presents the different outcomes for the cultured oocytes. The first picture in each series (Figures 20A, 20C, 20F, 20I) shows the preantral follicle before it is placed into the culture tube. The second picture in each series (Figures 20B, 20D, 20G, 20J) displays the oocyte retrieved from the follicle shown in the first frame. These oocytes have yet to be cultured in the IVF media. The bottom 3 outcomes have an additional micrograph (Figures 20E, 20H, 20K) showing the oocytes after a 6-hour culture in the IVF media.

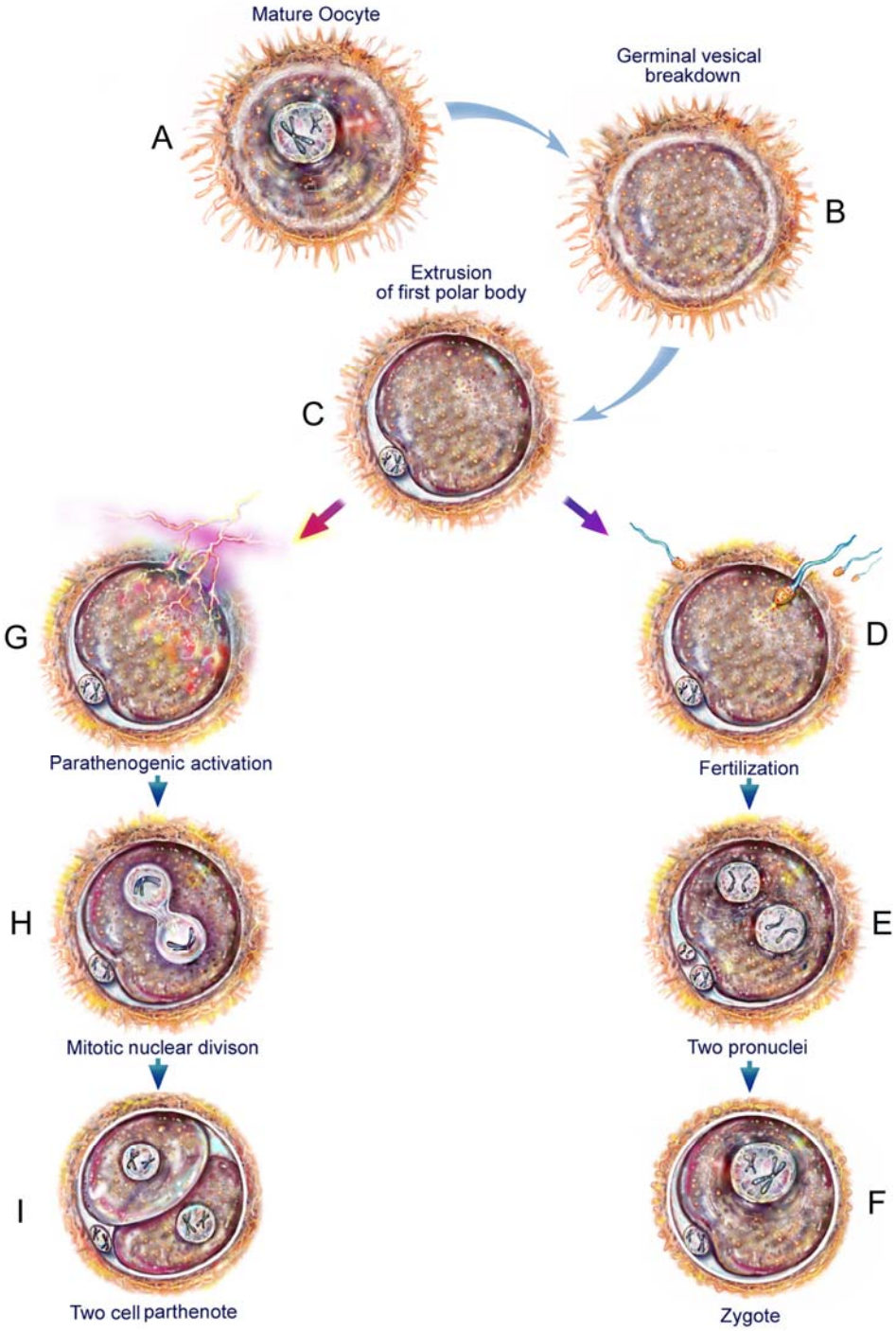


Figure 19: Meiotic stages of the ovulated oocyte and its subsequent progression through either fertilization or parthenogenesis. **(A)** Mature ovulated oocyte. **(B)** Germinal vesicle breakdown, a process in which the nucleus dissolves in preparation for the completion of meiosis occurs. **(C)** After the first meiotic division, the first polar body is extruded. The oocyte will arrest at this stage until it is fertilized or stimulated to undergo parthenogenesis. **(D)** Fertilization occurs and triggers the second meiotic division. **(E)** The second polar body is extruded and two pro nuclei, one from the ovum and one from the sperm, are present. **(F)** The two pro nuclei fuse into one nucleus, forming a zygote. **(G)** Parthenogenesis activation. (H) Activation triggers a mitotic nuclear division. **(I)** This is followed by cytokinesis and the formation of a two cell parthenote.

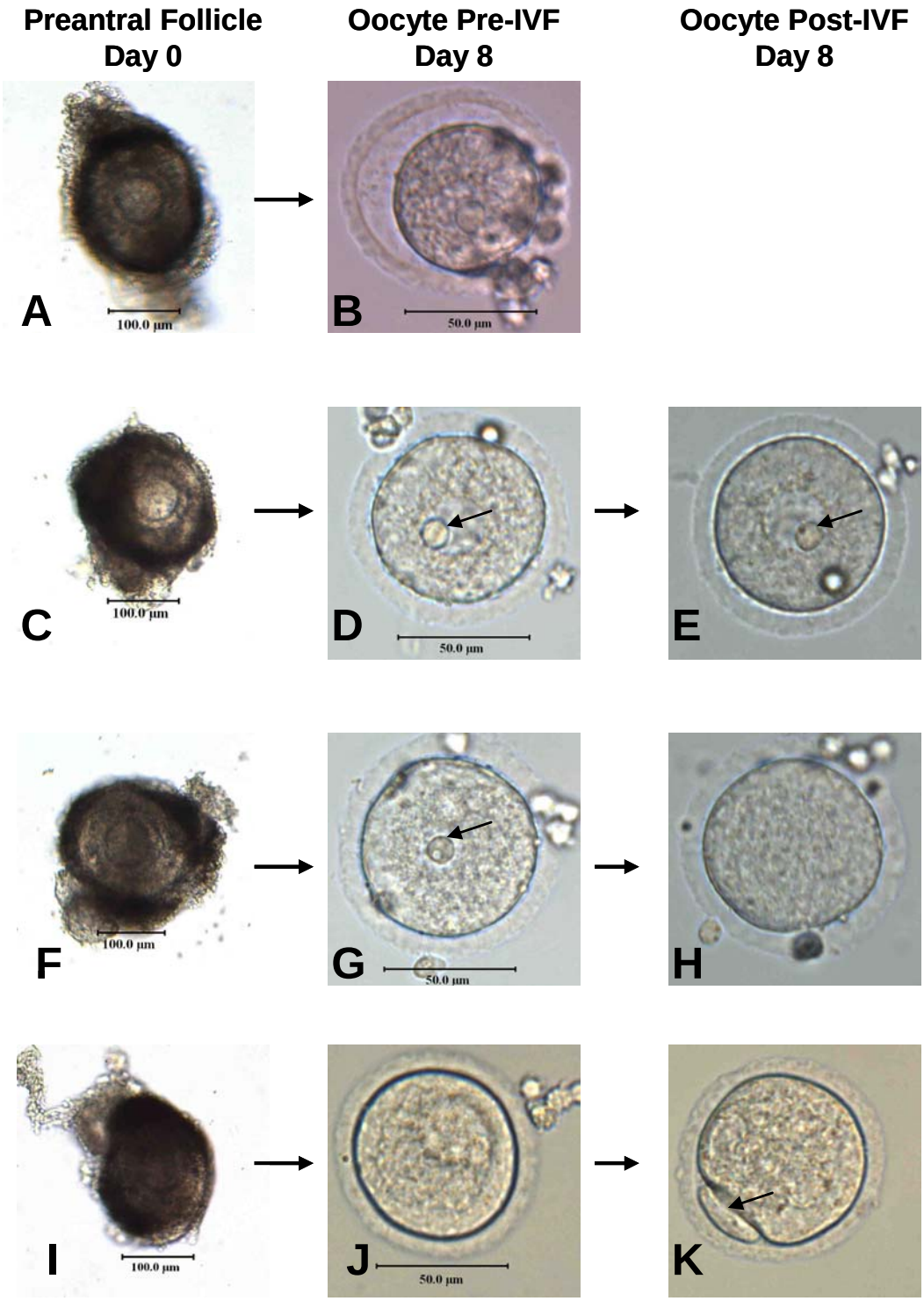


Figure 20: Micrographs of Follicles and Subsequent Oocytes. **(A, C, F, I)** Follicles on day 0. **(B)** Non-viable oocyte on day 7 from ambient oxygen group. **(D, G, J)** Viable oocytes on day 7 before culture in IVF-30 media. **(E)** Oocyte after culture in IVF-30 media, no germinal vesicle breakdown from ambient oxygen group. **(H)** Oocyte after culture in IVF-30 media, germinal vesicle breakdown from dynamic oxygen group. **(K)** Oocyte after culture in IVF-30 media, germinal vesicle breakdown and extrusion of the first polar body from dynamic oxygen group.

4.3.1 *In Vivo* Control

The superovulation protocol yielded a total of 19 oocytes that were observed for viability and maturation. Of the 19 oocytes recovered, all 19 were viable. After culturing the oocytes in the IVF medium, 18 (95%) went through germinal vesicle breakdown and extruded the first polar body (Figure 21). The 18 oocytes were then subjected to the parthenogenetic activation protocol. Of the 18 oocytes, 15 (83%) were activated (Figure 22).

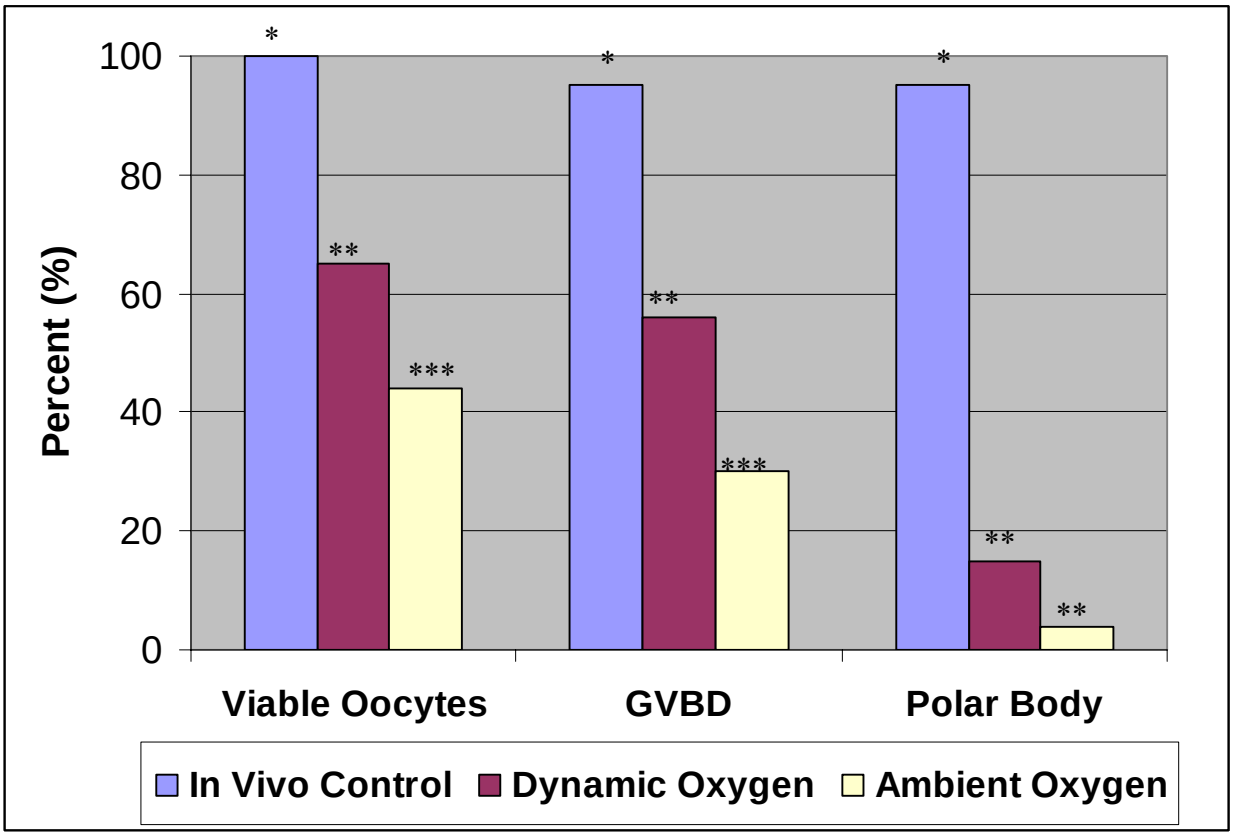


Figure 21: Oocyte Viability and Maturation Data (*: $p<0.05$).

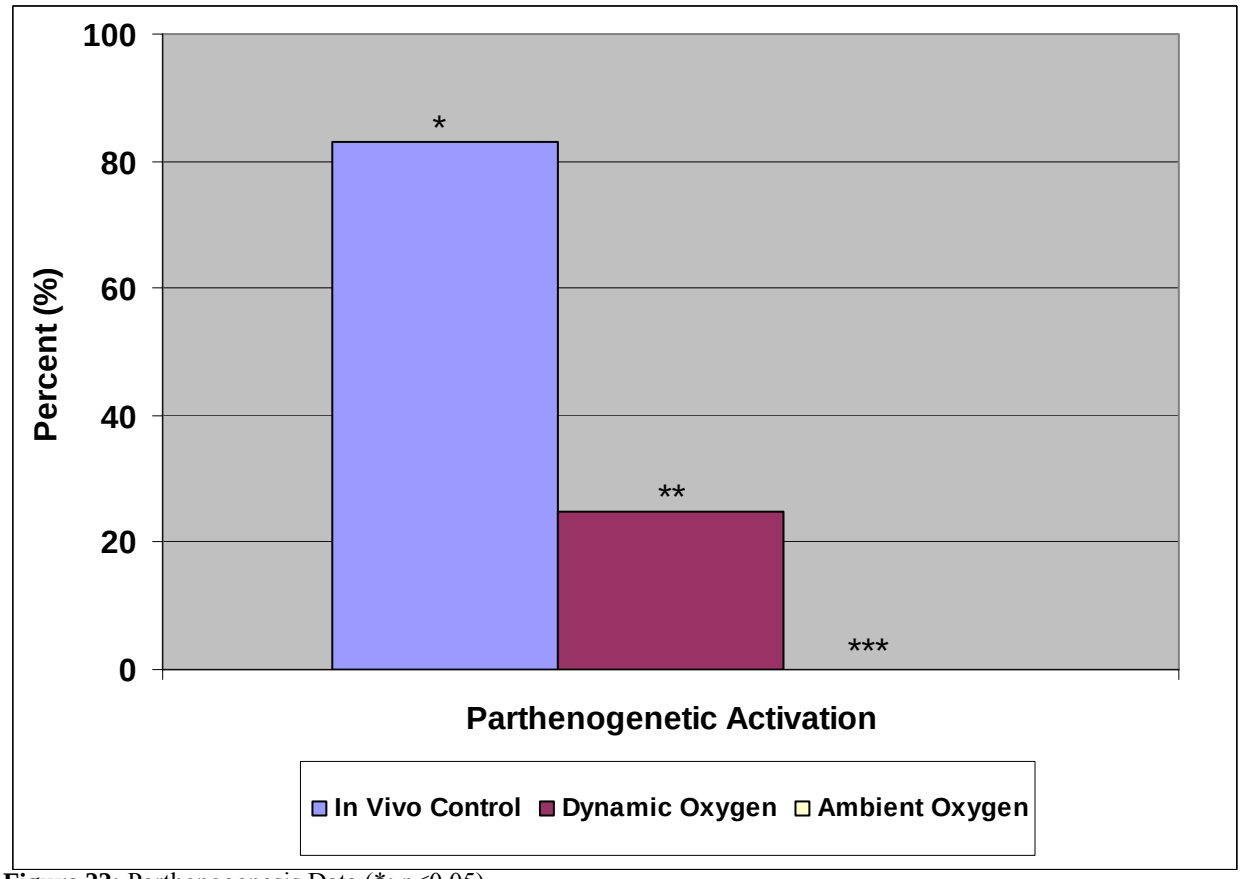


Figure 22: Parthenogenesis Data (*: $p<0.05$).

4.3.2 Ambient Oxygen Culture

In an attempt to develop a long-term culture system, immature ovarian follicles were cultured for 7 days in the suspension culture bioreactors under ambient oxygen tension. Figures 20A and 20B represents the outcome in which the oocyte has not survived the 7-day culture. Figure 20B is a non-viable oocyte that had retreated from the zona pellucida leaving a sizable portion of the perivitelline space empty. Of the 50 oocytes cultured in the ambient oxygen environment, only 22 (44%) remained viable.

The 22 surviving oocytes from the viability study were placed into IVF-30 media to determine their efficiency at resuming meiosis. After 6 hours in the IVF-30 media, germinal

vesicle breakdown was observed in 15 of the oocytes. Figures 20G and 20H portray a viable oocyte that went through germinal vesicle breakdown. The nucleus can clearly be seen in Figure 20G (arrow), but is no longer present after 6 hours in IVF-30 media (Figure 20H), signifying the resumption of meiotic maturation. This is in contrast to the outcome in Figures 20D and 20E in which the oocyte failed to undergo germinal vesicle breakdown as the nucleus is present (arrows) both before (Figure 20D) and after (Figure 20E) culture in the IVF-30 media. Ultimately, the ambient oxygen group produced 2 oocytes that extruded the first polar body. Figures 20J and 20K show an oocyte that not only went through germinal vesicle breakdown, but also extruded the first polar body (arrow).

From this experiment, it was found that the majority of the oocytes retrieved from the ambient oxygen group neither survived the culture nor resumed meiosis. Compared to the *in vivo* control, there was a significant decrease in all three of the observed parameters, the proportion of oocytes that were viable, underwent germinal vesicle breakdown, and extruded the first polar body (Figure 21). Although the yield of mature oocytes was low using this culture condition, we wanted to determine if the oocytes generated from the ambient oxygen environment could be parthenogenically activated. With the successful activation of these oocytes, we would take a first step toward the derivation of embryonic stem cell lines from *in vitro* matured preantral follicles.

In addition to being a source of fertilizable oocytes for the restoration of fertility, this study also examined whether *in vitro* matured oocytes could potentially be used to derive embryonic stem cell lines through parthenogenetic activation. Parthenogenesis is a form of asexual reproduction in some species in which the oocyte develops without ever being fertilized (Figure 19G, 19H, 19I). A number of methods have been developed to induce the

parthenogenetic activation of mammalian eggs. These eggs will divide mitotically progressing to the blastocyst stage, complete with an inner cell mass consisting of embryonic-like stem cells.

Embryonic stem cell lines have already been derived from the unfertilized oocytes of non-human primates [106, 107]. In the case of human eggs, it has been shown that parthenogenesis can be induced through several different methods [108, 109]. These activations have resulted in the acquisition of pluripotent stem cells from the resulting blastocyst [109]. Although promising, the primate and human studies have been performed with *in vivo* matured oocytes. However, it has been found that preantral mouse follicles can be cultured to produce an oocyte capable of establishing an embryonic stem cell line from parthenogenesis [5]. In this study, the issue of culturing larger follicles from more complex species for the purpose of producing oocytes capable of undergoing this activation was addressed.

In order to assess whether our culture method could produce the mature oocytes necessary to attain these stem cells, a number of oocytes from the ambient oxygen group were subjected to an activation protocol utilizing the chemical induction agent, strontium. A total of 22 oocytes from the ambient oxygen group were put through the parthenogenetic activation protocol. Of the 22 oocytes none were activated.

Unfortunately, the oocytes from the ambient oxygen group were unable to activate. It was determined that the functional capability of the oocytes cultured at higher oxygen tensions is significantly diminished compared to mature oocytes that activate in response to strontium exposure [110]. This suggested that the parameters for this culture condition needed to be altered in order to develop a successful long-term culture technique that could produce mature oocytes with the same potential as those matured *in vivo*. We hypothesized that if the oxygen tension in the incubator was decreased to *in vivo* levels for developing preantral follicles, the

result would be an increase in the proportion of mature functioning oocytes obtained from the culture.

4.3.3 Low Oxygen Culture

The preliminary low oxygen experiments focused on the effect that this environment had on the morphology of the preantral follicles. Hemotoxylin and eosin stained sections were compared between follicles cultured for 7 days at 2% oxygen tension (Figure 23A and 23B) and 15% oxygen tension (Figure 23C and 23D). The low oxygen sections show that the tissue structure is intact and the cells are not necrotic, which is a concern when culturing at such low levels of oxygen. Follicles grown in the high oxygen environment had ruptures in the basement membrane, compromising the three dimensional integrity of the tissue along with a loss of cell junctions creating empty gaps around the oocyte. From these observations, it was found that not only could preantral follicles survive in a low oxygen environment, but also their three dimensional structure more closely resembled in situ follicle structure. However, a static low oxygen tension is also not what developing follicles experience *in vivo*. We further hypothesized that for long-term cultures designed to support follicle maturation, a method of oxygen delivery that would increase over the duration of the culture period should be implemented.

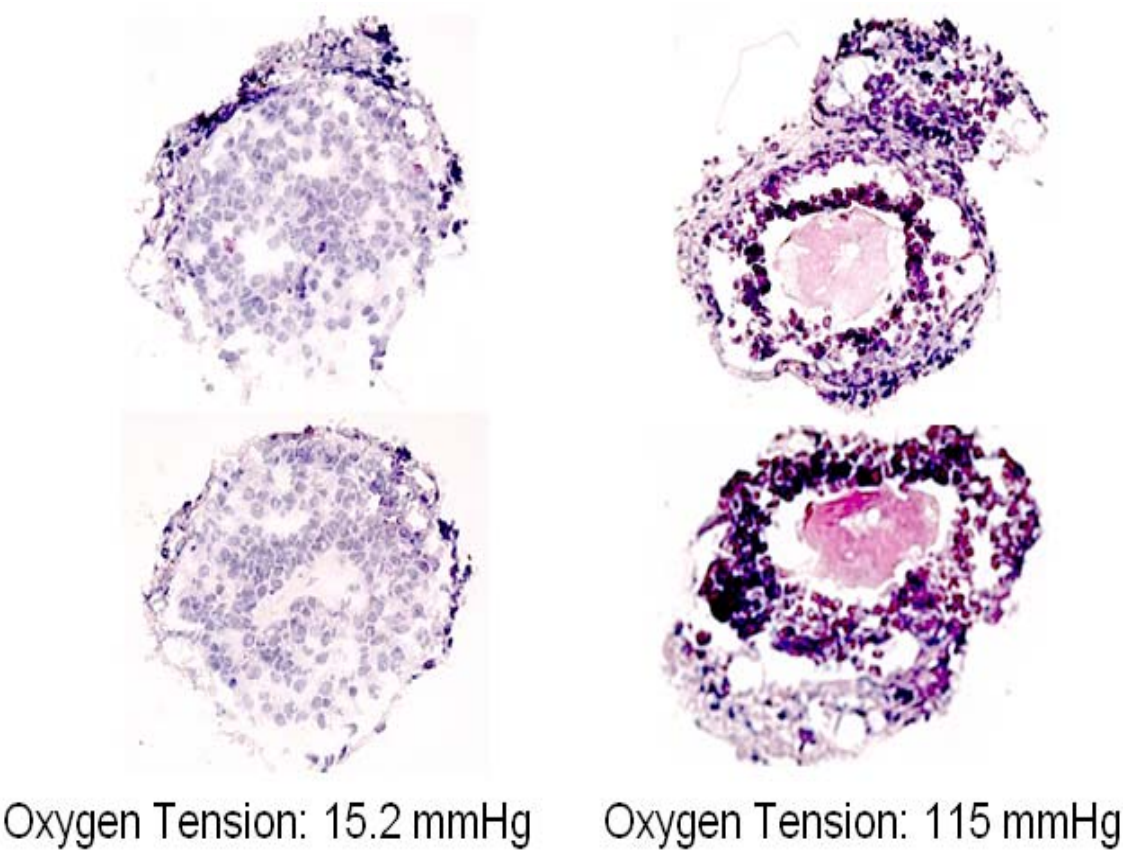


Figure 23: Hemotoxylin and eosin stained sections of follicles cultured for 7 days in **(A, B)** low oxygen tension (2%) or **(C, D)** high oxygen tension (15%).

4.3.4 Dynamic Oxygen Culture

Utilizing *in vivo* oxygen tensions to enhance tissue function during *in vitro* culture has been used in a number of related fields. It has been shown that maturing porcine oocytes in a low oxygen environment improves not only their ability to parthenogenetically activate but also the quality of the resulting blastocysts [111]. It was also determined that culturing embryos at an oxygen tension characteristic of levels found in the oviduct and uterus improved both the establishment and maintenance of murine stem cells [112]. It is becoming evident that the fields of follicular development, oocyte maturation, and embryo culture are benefiting from this trend to mimic the

unique *in vivo* metabolic needs of these avascular tissues. In this spirit, several groups have examined the effects of lower oxygen tensions on follicle growth and development [113-115]. It was found that by culturing preantral follicles under 5% oxygen, there was a significant increase in oocyte survival [113, 115]. Although these studies use oxygen tensions that are more suitable for early follicle development, the oxygen concentrations are still held constant for the duration of the culture periods. To better mimic the native oxygen environment, the oxygen concentrations for the dynamic oxygen protocol implemented in this study were based on the unique *in vivo* realities experienced by maturing follicles throughout their life-cycle in the ovary.

For the purposes of our study, this dynamic oxygen protocol was then combined with the use of bioreactors that enable follicles to grow without flattening [79]. As in the ambient oxygen culture, immature follicles were cultured for 7 days at which point the oocytes were retrieved and observed for viability, germinal vesicle breakdown, and polar body extrusion. Of the 54 oocytes cultured in the dynamic oxygen environment, 35 (65%) were viable. After 6 hours in the IVF-30 media, germinal vesicle breakdown was observed in 30 (56%) oocytes from the dynamic oxygen group, with 8 of those oocytes extruding the first polar body.

Although these figures are still significantly less than the *in vivo* control values, significant increases were observed in the dynamic oxygen group for both the proportion of oocytes that remained viable and the proportion of oocytes that resumed meiosis compared to the ambient oxygen group (Figure 21). The dynamic oxygen environment not only permitted the development of mature oocytes, but significantly increased the number of oocytes that resumed meiosis by undergoing germinal vesicle breakdown, showing that both the quantity and quality of the oocytes were enhanced using this culture method. Furthermore, it was determined that there was no significant difference ($p>0.05$) in oocyte diameter between the *in vivo* control,

70.4+/-5.6 µm, the dynamic oxygen, 68.5+/-2.5 µm, and the ambient oxygen, 68.8+/-2.7 µm groups. We next wanted to determine if the oocytes generated from the dynamic oxygen environment could be parthenogenically activated.

Utilizing the chemical inducing agent strontium, a total of 28 oocytes from the dynamic oxygen group were put through the parthenogenetic activation protocol. Of the 28 oocytes cultured in the dynamic oxygen environment, 7 were successfully activated. This is a significant increase when compared to the ambient oxygen group that failed to produce a single oocyte capable of activation (Figure 22). Figure 24 shows the micrograph of an activated oocyte and its corresponding Hoechst-stained image. Figure 24A shows the activated oocyte at the 2-cell stage while Figure 24B is the fluorescent image of the oocyte with the nuclear-staining Hoechst dye confirming the presence of a nucleus in each of the 2 cells.

Figure 24: (A) Micrograph of Activated Oocyte (400x) (B) Hoechst-stained Activated Oocyte (400x).

The derivation of embryonic stem cells from these parthenotes is beyond the scope of this paper. However, by activating these eggs, we confirmed that the oocytes retrieved from this

improved culture environment have the potential to become a source for embryonic stem cells. Furthermore, this indicates that culturing preantral follicles in a high oxygen environment will limit the functional potential of the oocytes.

4.3.5 Intracytoplasmic Sperm Injection

From the oocyte viability and maturation studies, it was found that the long-term culture of preantral ovarian follicles for the purpose of producing mature oocytes can become more efficient by altering the oxygen environment to mimic the native ovarian environment. However, in order to prove that this culture technique can ultimately be utilized as a method to restore fertility, the fertilization of a cultured oocyte must be demonstrated.

In this study, 8 oocytes from the dynamic oxygen group were injected with sperm from a mature male Sprague-Dawley rat. Of these 8 oocytes, 2 pro nuclei (2pn) were observed in 2 of the oocytes within 24 hours. Figure 25A shows one of the 2pn oocytes while Figure 25B is the fluorescent image of the oocyte with the nuclear-staining Hoechst dye confirming the presence of both pro nuclei in the egg. Neither of these eggs progressed to the 2-cell stage (Figure 19F), however, the presence of both pro nuclei in the egg confirm the fertilization (Figure 19E).

By fertilizing these oocytes, it gives us the proof of concept that this dynamic oxygen culture technique is a potential option to restore fertility in females who undergo sterilizing therapy. As this project moves forward, we will look to improve the ICSI procedure and the media used to culture the embryos in order to support the embryo to later stages of development.

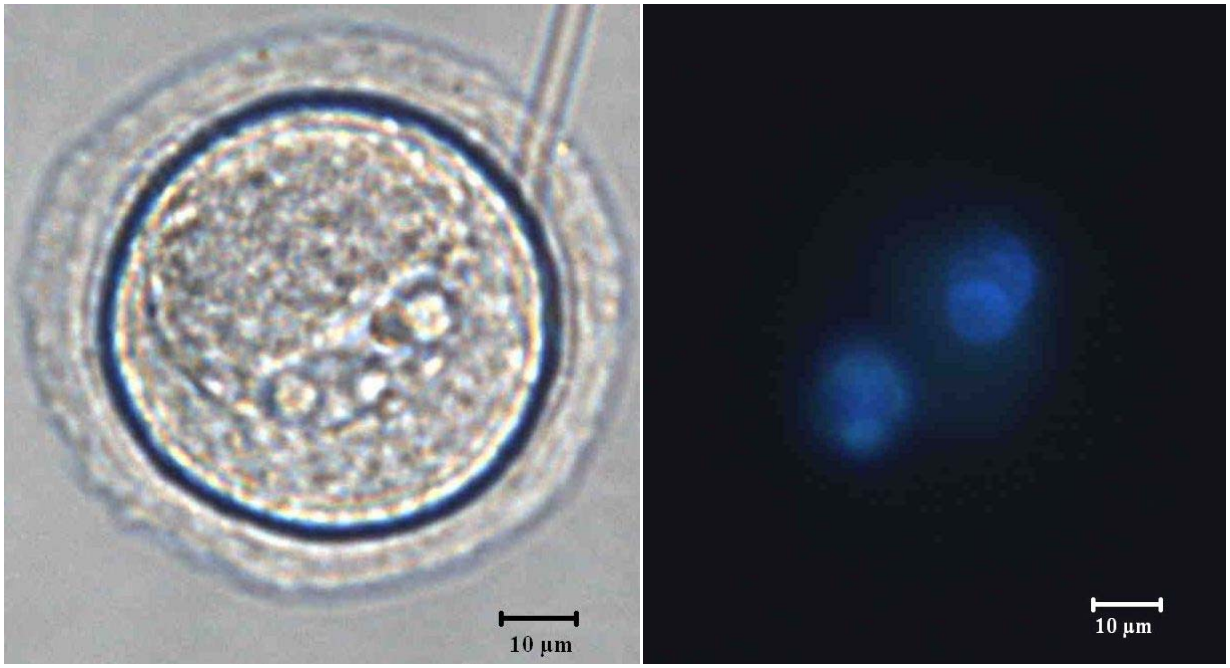


Figure 25: (A) Micrograph of Fertilized Oocyte (400x). Arrows indicated the 2 pro nuclei. (B) Hoechst-stained Fertilized Oocyte (400x).

4.4 CONCLUSIONS

The dynamic oxygen environment produced a significantly higher yield of healthy oocytes than the static control. More importantly, the oocytes from the dynamic oxygen environment were significantly more efficient at progressing through germinal vesicle breakdown than their compliments in the control. In conclusion, these data demonstrate that both the yield and quality of the oocytes derived from *in vitro* cultured preantral follicles can be improved by utilizing a dynamic oxygen protocol designed to mimic the *in vivo* environment of the ovary. Furthermore, it was found that not only can immature rat preantral follicles be cultured to produce oocytes capable of parthenogenetic activation, but the activation was only achieved when the oocyte had been cultured in the dynamic oxygen environment. Finally, the *ex vivo* ovulated oocytes can be fertilized by ICSI and if we are able to demonstrate a similar approach with human follicles, then

dynamic oxygen culturing would provide hope to young children who must sacrifice fertility for life.

5.0 THE EFFECT OF OXYGEN CONCENTRATION ON PREANTRAL FOLLICLE GENE EXPRESSION

5.1 INTRODUCTION

In our previous study (Chapter 4), it was found that the utilization of a dynamic oxygen protocol during the culture of preantral follicles significantly increased the proportion of follicles that remained viable, resumed meiosis, and parthenogenetically activated compared to those follicles cultured at the standard ambient oxygen pressure of 20% (152 mmHg). The dynamic oxygen protocol was modeled after the *in vivo* oxygen environment of the native ovary, beginning with the initial oxygen tension in the incubator set at 4% (30.4 mmHg). In order to mimic the *in vivo* transition from avascular to vascular oxygen levels, the oxygen tension was increased 1% every 24 hours until the end of the 7 day culture period with a final oxygen tension of 11% (83.6 mmHg), a level within the normal range for arterial oxygen.

We believe that the positive results observed from the dynamic oxygen group are attributed to an environment that better mimicked that experienced by preantral follicles in the native ovary. In contrast, we feel that the damaging effect the high oxygen environment had on the ambient oxygen group is due to the drastic increase the preantral follicles experienced at the onset of the culture. By being placed in an environment more suitable to mature graafian follicles [99], the granulosa cells may not differentiate properly, leading to desynchronized

follicle development. Previous to this study, no group has analyzed global gene expression patterns of preantral follicles cultured in varied oxygen environments and compared them to the gene expression patterns of native preantral follicles

In this study, gene chip analysis was performed on three groups of follicles, an *in vivo* control, a three-day high oxygen group, and a three-day low oxygen group. The objective was to compare gene expression of the *in vivo* control group to both the low and high oxygen culture groups. Within this analysis, the desire was to determine which experimental groups' gene expression profile was more similar to that of the control and which genes and pathways were altered for each of the experimental groups as they pertain to the control.

5.2 MATERIALS AND METHODS

5.2.1 Animals and Ovarian Dissection

All animal experiments were performed in accordance with National Institutes of Health guidelines and with institutional approval. Sprague-Dawley rats were obtained from Hilltop Lab Animals (Pittsburgh, PA) and housed under standard conditions. The animals were sacrificed by CO_2 exposure and cervical dislocation. Ovaries were dissected and placed immediately in warmed culture medium, consisting of Leibovitz L-15 Medium (Gibco BRL). The follicles were then mechanically dissected from the ovary using a pair of syringes with 26 gauge needles. All follicles used in the experiments were measured in two dimensions, using an inverted microscope fitted with an ocular micrometer. Only intact follicles that were between 140 and 170

microns in diameter were used in culture. Intact follicles between 200 and 250 microns in diameter were used as the *in vivo* control group.

5.2.2 Follicle Culture

Culture media consisted of α-Minimal Essential Medium (Gibco BRL, Invitrogen Corporation, Grand Island, NY) with additives of 8-bromo-cGMP (5 mM), ITS+ (1% solution of insulin, 10 mg/L; transferrin, 5.5 mg/L; linoleic acid, 4.7 mg/L; selenium, 5 mg/L), Pen/Strep (1%, penicillin 100 U/ml, streptomycin 100 μg/ml), all from Sigma Chemical Co. (St. Louis, MO), and recombinant Follicle Stimulating Hormone, rFSH (0.7 IU/mL), (Serono Laboratories, Geneva). Culture media was placed into 12 x 75 mm polypropylene culture test tubes (500 μl/tube) and cultured in 5% CO_2 and 37 ^{0}C humidified incubator.

Suspension culture was attained by placing the 6 ml culture tubes in a circular rotator plate (Glas-Col, Terre Haute, IN), having a diameter of 30.5 cm, which was rotated around its horizontal axis at rate between 8-15 rpm. Therefore, as the plate rotates, the tubes slowly orbit the axis of the plate.

The incubators used for this study were both CO_2 and O_2 controlled. For the ambient oxygen group, the oxygen tension was cultured in the traditional static oxygen tension of 20%. For the low oxygen group, the oxygen tension was set at 4%. Both groups were cultured for three days. The third group in this study was the control group comprised of *in vivo* follicles. These follicles were excised from the ovary with diameters between 200 and 250 microns. Instead of being placed into culture, this group immediately went through the RNA isolation protocol.

5.2.3 RNA Isolation

Follicles of the same sample group were placed into 1mL of Trizol reagent, passing the lysate several times through a pipette. The lysate was then incubated for 5 minutes at 30^{o}C and transferred to an RNase-free tube. 0.2 mL chloroform and 10ug glycogen were then added to the trizol reagent, shaken vigorously for 15 s, and incubated at room temperature for 3 minutes. Next, the reagent was centrifuged at 11,000 rpm for 15 minutes at 2^{o}C. The upper phase of the resulting solution was transferred into a fresh tube and mixed with 250 uL of isopropyl alcohol. This was again incubated for 10 minutes at 30^{o}C and centrifuged for an additional 10 minutes at 11,000 rpm. The RNA pellet was then washed with 75% ethanol and centrifuged at 7500 rpm for 5 minutes before being air dried for 10 minutes. Finally, the RNA pellet was redissolved in 20 uL of diethyl pyrocarbonate treated water and incubated at 59^{o}C for 10 minutes.

5.2.4 RNA Quality

Verifying the integrity of the RNA starting material is crucial for proper gene expression analysis. In this study, each sample was tested using the Agilent 2100 bioanalyzer. The bioanalyzer uses electrophoretic separation to separate the RNA sample and then detect it using laser induced fluorescence detection. The software then creates an electropherogram that provides a visual assessment of the quality of the RNA sample along with the ratio of the 18S and 28S ribosomal subunits.

In order to more accurately interpret these electropherograms, the RNA Integrity Number (RIN) was developed [116]. The RIN software algorithm ranks RNA on a scale from 1 to 10, 1 being most degraded and 10 being intact (Figure 26).

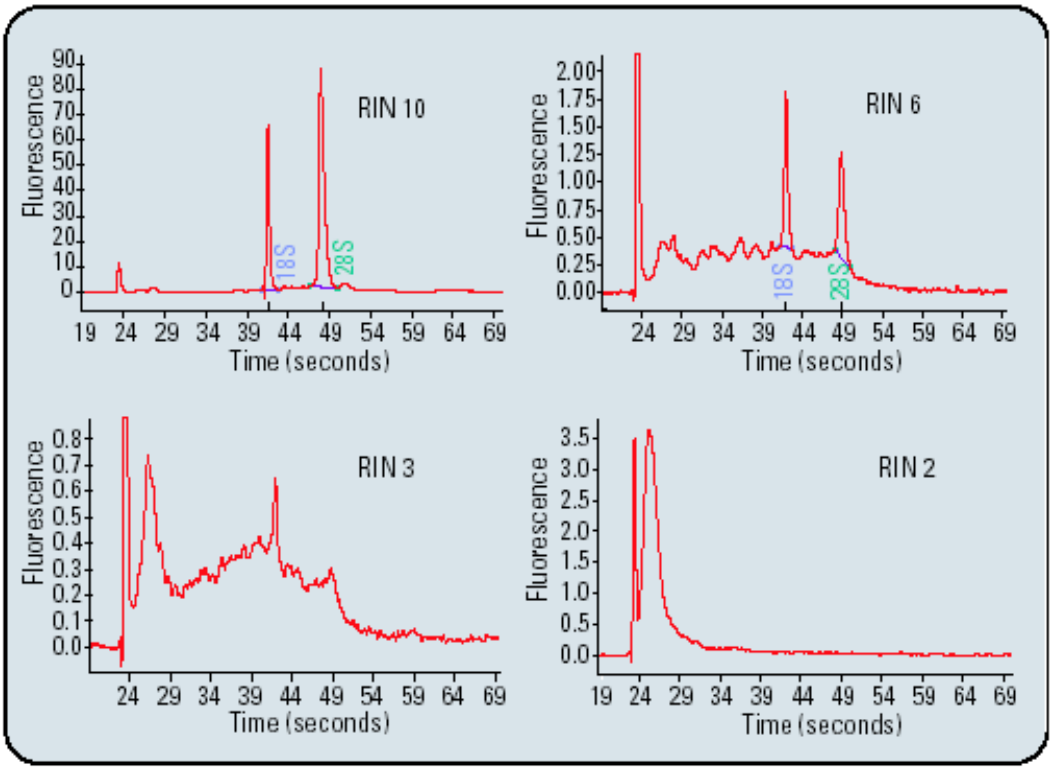


Figure 26: Sample electropherograms used to train the RNA Integrity Number (RIN) software. Samples range from intact (RIN 10), to degraded (RIN 2). Reprinted from [116]

Ideally, the electropherogram will have a low baseline absent of any signal with sharp ribosomal peaks. This algorithm analyzes the important areas of the entire electropherogram for proper signal areas, intensities, and ratios. These areas include the pre-, 5S-, fast-, inter-, precursor-, and post regions along with the marker, 18S, and 28S peaks (Figure 27). For the RNA to be considered to have the appropriate integrity for gene expression analysis, the minimum desirable RIN is 6.0.

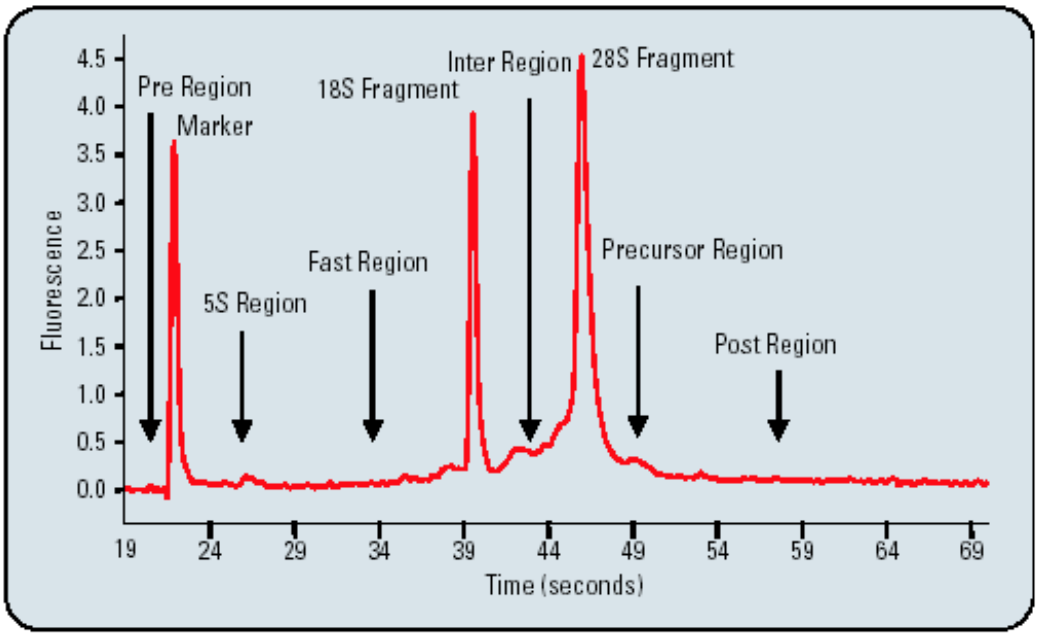


Figure 27: Electropherogram detailing the regions that are indicative of RNA quality. Reprinted from [116]

5.2.5 RNA Amplification

RNA samples were prepared using the Illumina® Totalprep RNA Amplification Kit from Ambion according to manufacturer's instructions [117]. Briefly, 500 ng total RNA was used to prepare ds-cDNA using T7-oligo(d)T primer. Reverse transcription was followed by *in vitro* transcription in the presence of biotinylated nucleotides (Figure 28).

Biotinylated cRNA samples were hybridized to RatRef-12 Expression Beadchip arrays in a proprietary hybridization buffer overnight at 58°C on a platform rocker. Following the 18 hour hybridization, arrays were washed in proprietary buffers to remove excess labeled cRNA. Florescent tagging was achieved by incubation with Cy3-streptavidin for 10 min at RT. Excess dye solution was removed with further buffer washes. Slides were dried by low speed centrifugation and scanned in the Illumina BeadScanner. Resulting image files were analyzed using Bead Studio version 3.2.3.

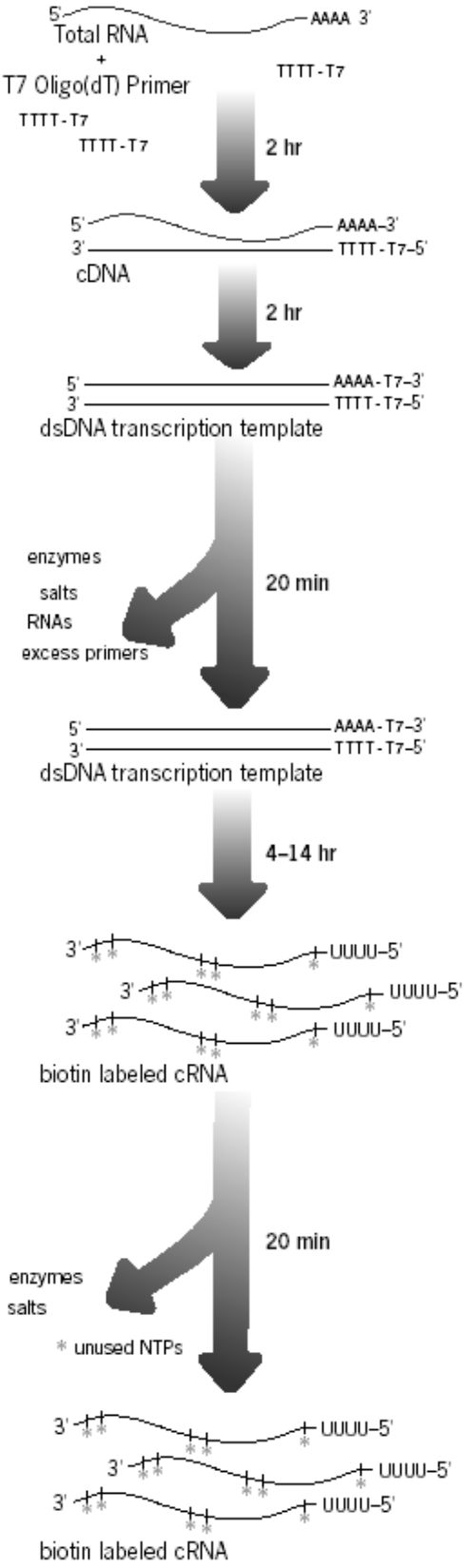


Figure 28: RNA Amplification Procedure. Reprinted from [117]

5.2.6 Experimental Design

The study design for analyzing the samples is depicted in Figure 29. For the *in vivo* control, high oxygen and low oxygen groups, the expression value for each gene is the average gene expression value from three separate samples analyzed on three different microarrays.

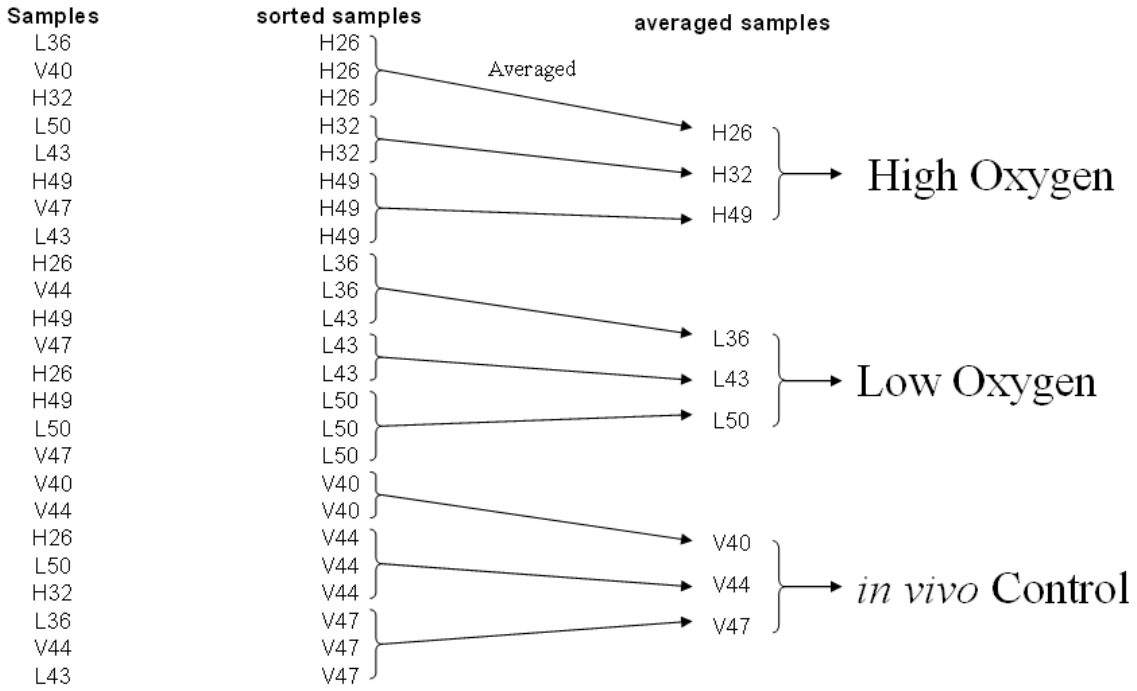


Figure 29: Data Sample Design.

5.2.7 Normalization

When processing RNA for gene chip analysis, systematic variations between samples can occur, resulting in errors that hinder proper expression value comparison. These variations are due to factors such as concentration differences in a processing solution or inconsistent hybridization temperatures [118]. In order to improve the validity of the data and ensure that variations in gene expression levels are biological in nature, normalization methods that factor out these

systemic errors have been developed. In this study, the gene expression values were normalized using four different normalization algorithms: average, rank invariant, cubic spline, and quantile.

The average method regulates the intensities of two samples of gene array values such that their means become equal [118]. This is a simple algorithm that determines a normalization factor by dividing the mean of sample A with the mean of sample B and then scaling all the gene expression values in the sample with the lower mean by the determined factor.

The rank invariant method determines the scaling factor by only dividing the average of rank invariant genes instead of every gene. These rank invariant genes are genes that have expression values that appear in a regular order compared to other genes within the sample [118]. By using this method, outliers are not used to determine the scaling factor. The underlying assumption of this method is that there exists a subpopulation of genes in the samples being compared that will not be differentially expressed [118]. Therefore, samples from different tissues most likely could not be normalized using this method.

The cubic spline algorithm is non-linear. With this method, the array data is divided into a group of quantiles. Then the average for each quantile in sample A is divided by the average of the corresponding quantile in sample B, resulting in a different scaling factor for each quantile [118]. Each of these factors is then used to scale only the genes within that specific quantile.

5.3 RESULTS AND DISCUSSION

5.3.1 Global Gene Expression Analysis

The initial gene expression analysis focused on the entire gene expression patterns for each group. Correlation plots comparing gene expression intensities between both the low and high oxygen groups vs. the *in vivo* control were analyzed for each normalization method, none (Figure 30A and 30B), average (Figure 31A and 31B), cubic spline (Figure 32A and 32B), rank invariant (Figure 33A and 33B), and quantile (Figure 34A and 34B). From these plots, R^2 values were obtained and compared to determine if either culture condition caused the preantral follicles to produce an overall gene expression pattern that more closely resembled the gene expression pattern from the *in vivo* control (Table 4).

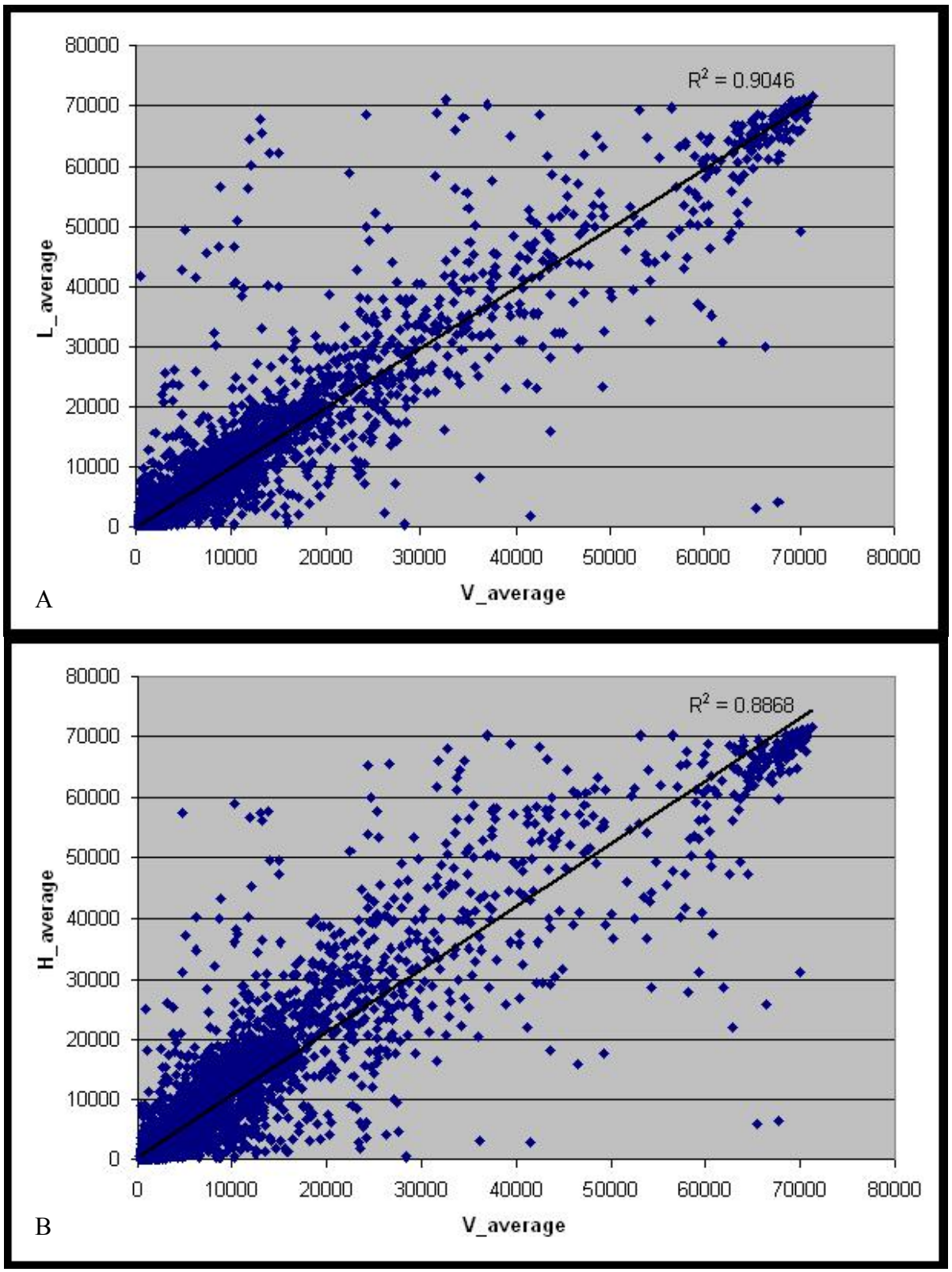
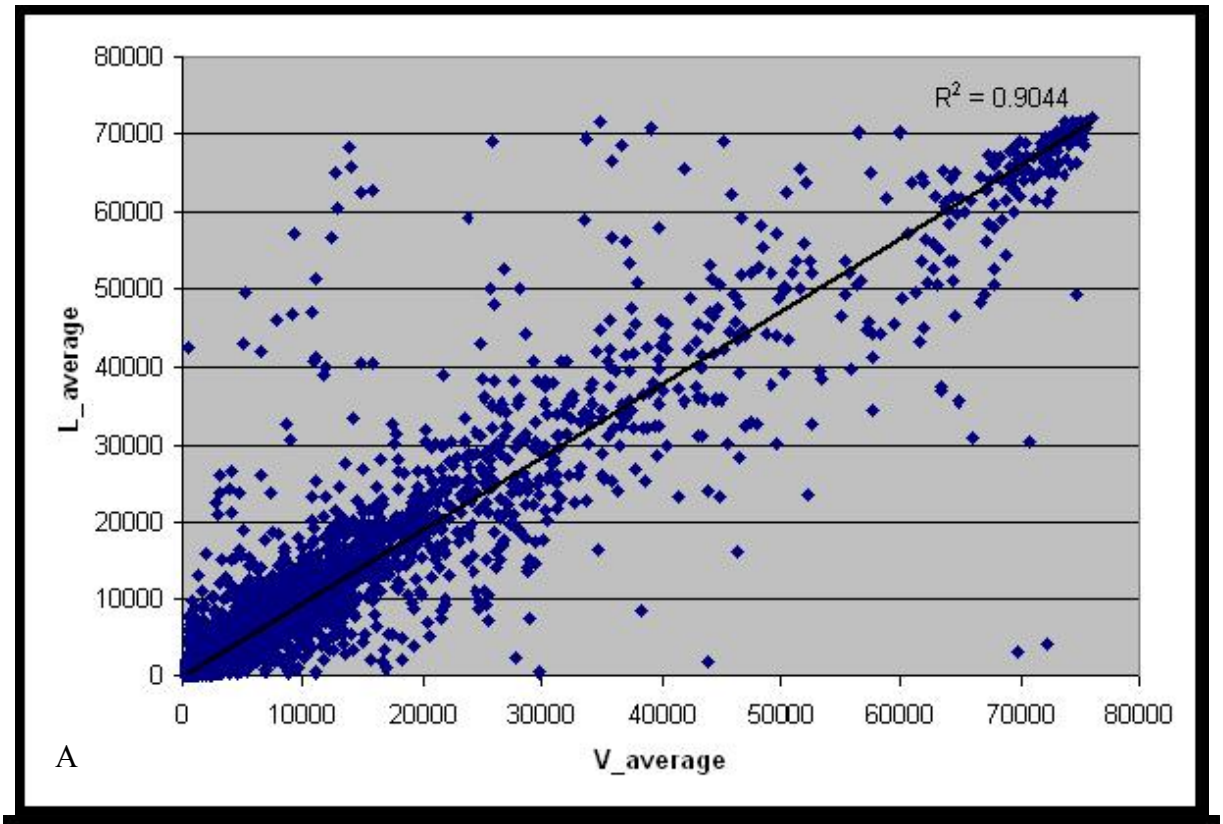


Figure 30: Global gene expression correlation plot (No normalization). (A) low oxygen group vs. *in vivo* control group (B) high oxygen group vs. *in vivo* control group.



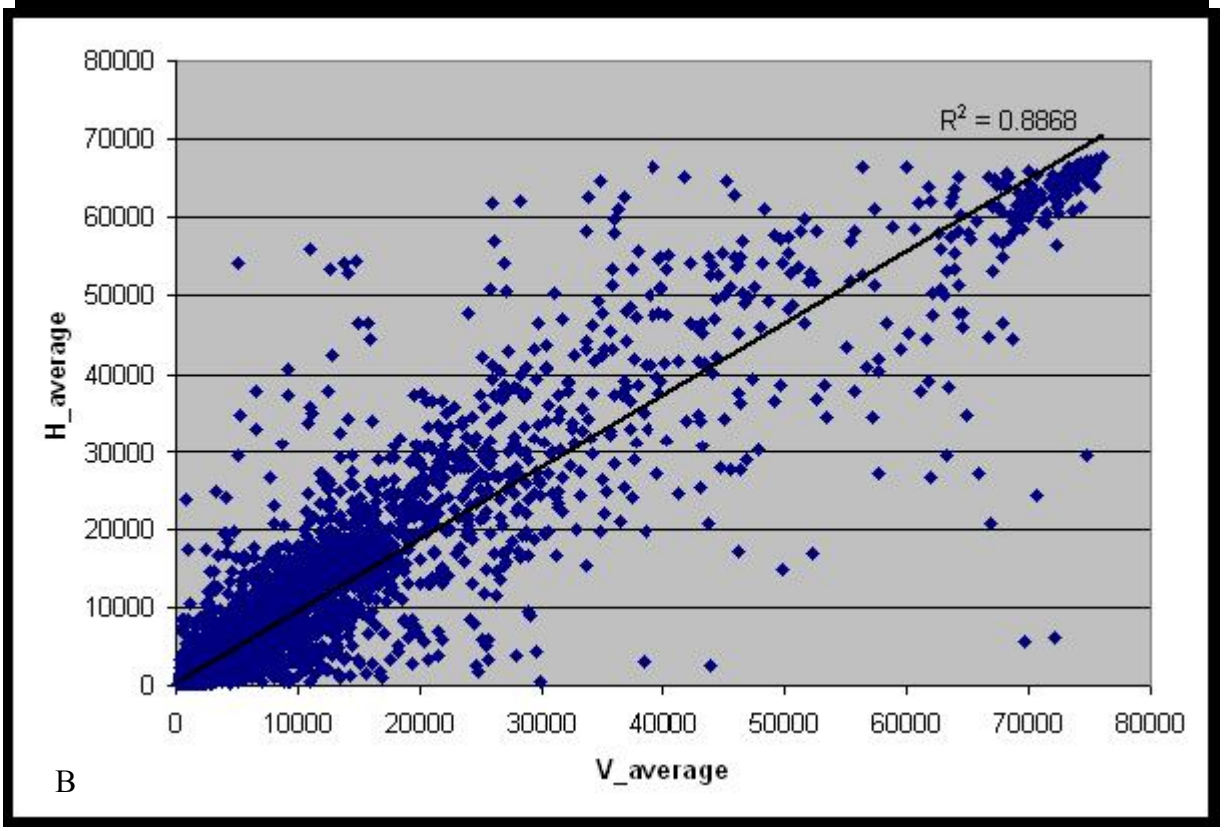


Figure 31: Global gene expression correlation plot (Average normalization method). (A) low oxygen group vs. *in vivo* control group (B) high oxygen group vs. *in vivo* control group.

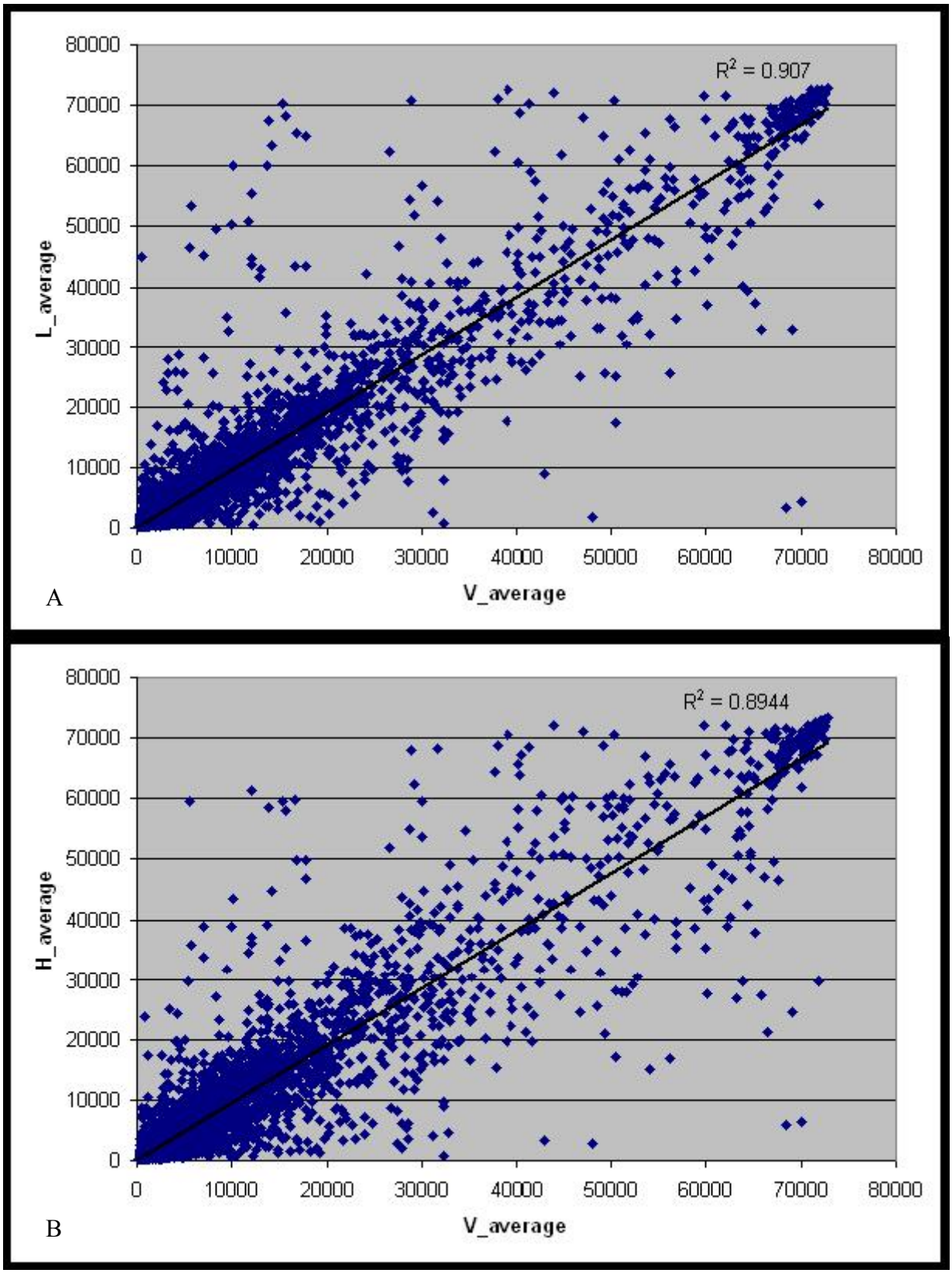


Figure 32: Global gene expression correlation plot (Cubic Spline normalization method). (A) low oxygen group vs. *in vivo* control group (B) high oxygen group vs. *in vivo* control group.

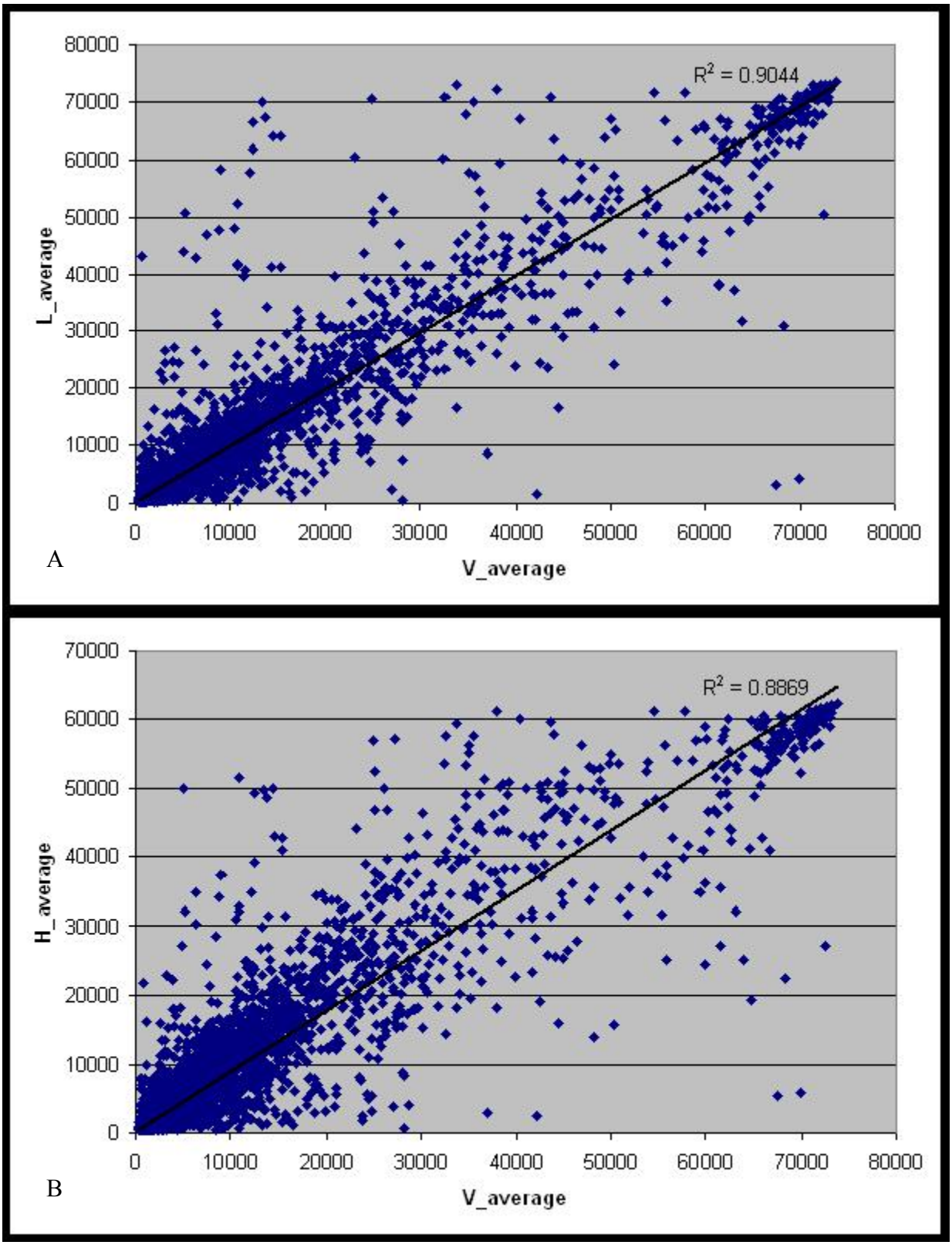


Figure 33: Global gene expression correlation plot (Rank Invariant normalization method). (A) low oxygen group vs. *in vivo* control group (B) high oxygen group vs. *in vivo* control group.

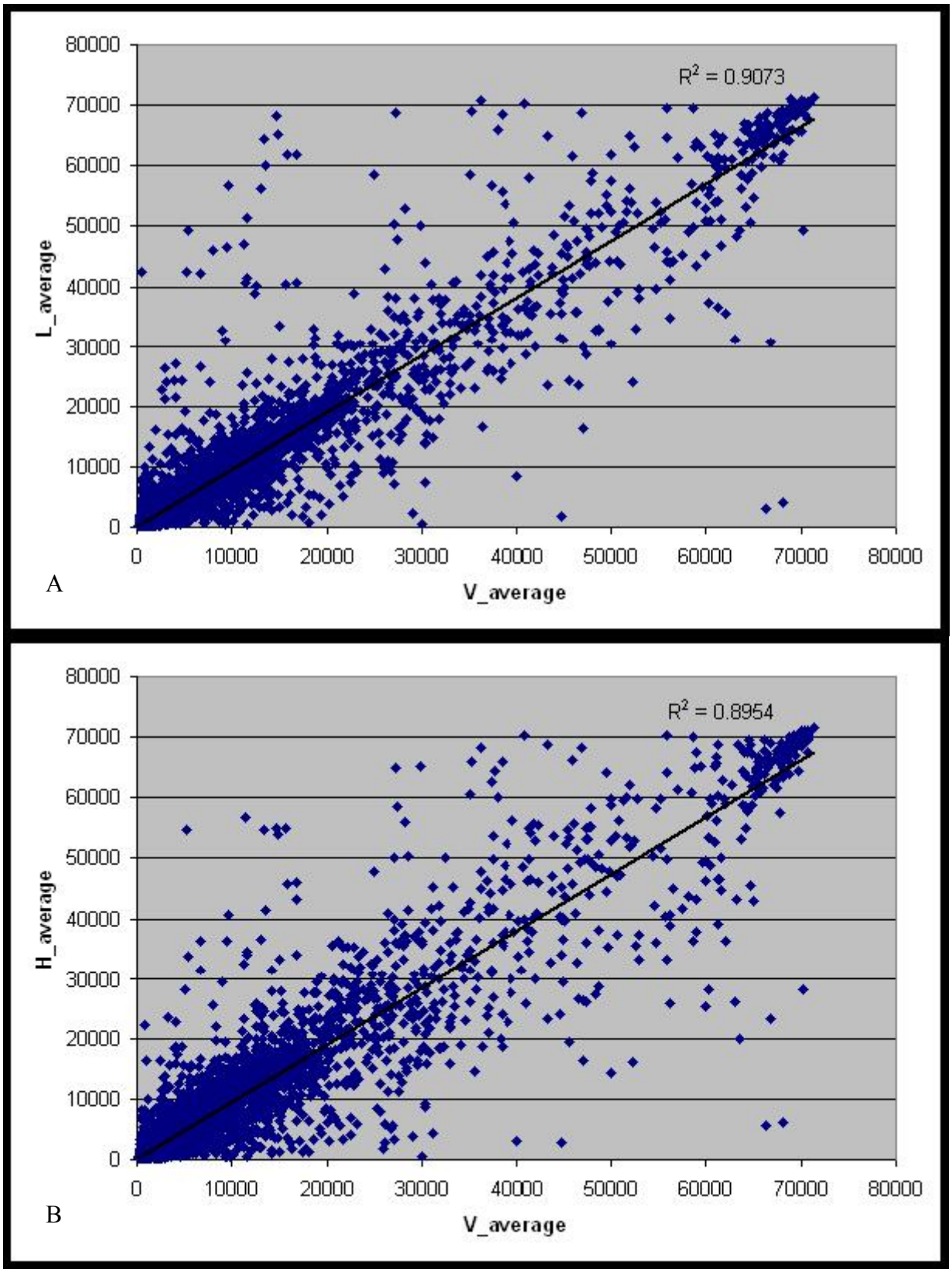


Figure 34: Global gene expression correlation plot (Quantile normalization method). (A) low oxygen group vs. *in vivo* control group (B) high oxygen group vs. *in vivo* control group.

Table 4: Summary of R2 Values from the Normalization Methods.

R^2	Low Oxygen Group VS. *in vivo* Control	High Oxygen Group VS. *in vivo* Control
No Normal	0.9046	0.8868
Average	0.9044	0.8868
Cubic Spline	0.9070	0.8944
Rank Invariant	0.9044	0.8869
Quantile	0.9073	0.8954

With the RatRef-12 expression beadchip containing over 22,000 probes designed to analyze the entire rat genome, a drastic difference between the low oxygen group and high oxygen group was not expected. However, from the R^2 values shown in table 4, the low oxygen group displays a better correlation to the *in vivo* control than the high oxygen group. Although the difference is small, it is consistent, remaining robust to several different normalization methods. For this reason, it is believed that this difference is due to biological variations within the cultured follicles rather than any systematic error in the processing of the samples [118].

5.3.2 Differentially Expressed Genes

Significant differences in R^2 values for whole genome correlation plots would most likely be observed in comparisons between different tissue types. When analyzing differences between samples of the same tissue, such as in this study, the differentially expressed genes are identified and then investigated in order to isolate subsets of genes that would affect proper tissue function.

Algorithms that can identify pathway differences for certain processes and disease states have been developed. However, both the low oxygen group and the high oxygen group in this study were expected to be relatively healthy, making the differences between the sample groups more subtle.

For the purpose of this study, data mining was performed to uncover the genes that were differentially expressed between the low oxygen group and the *in vivo* control (Table 5 and Figure 35) and between the high oxygen group and the *in vivo* control (Table 6 and Figure 36). These genetic variations were then analyzed to determine if any could have an adverse affect on preantral follicle development.

Follicular development during the preantral stage is controlled by several crucial processes within the follicle: the production of the proper differentiation and growth factors [96, 97] and the maintenance of appropriate levels of steroidogenesis and metabolism [119-121]. These processes synergistically regulate the growth and maturation of both the oocyte and the cells that support it [122].

Oocyte growth and maturation is controlled by nutrients and molecular signals originating from the surrounding granulosa cells [122]. Granulosa cells differentiate throughout the life of a follicle, producing and transporting different growth and maturation factors to the oocyte during the different stages of development. The rate at which these cells differentiate and the stage-dependent manner with which they secrete the appropriate factors ensure proper development. It is this interaction between the granulosa cells and the oocyte that may be the most crucial process to maintain throughout any long-term culture system [122]. Studies have shown that growth factors such as growth differentiation factor-9 not only initiate granulosa cell proliferation, but also regulate granulosa cell differentiation[122]. Therefore, in order to design a

culture system that properly supports oocyte growth and maturation, normal proliferation and differentiation of the granulosa cells must be maintained.

Table 5: Differentially expressed genes in the low oxygen group vs. the *in vivo* control

Rank	Gene	J5 score
1	**Rattus norvegicus hemoglobin alpha, adult chain 1 (Hba-a1), mRNA.**	-117.092
2	**Rattus norvegicus hemoglobin beta chain complex (Hbb), mRNA.**	-114.528
3	**PREDICTED: Rattus norvegicus similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (LOC295452), mRNA.**	100.867
4	**Rattus norvegicus lactate dehydrogenase A (Ldha), mRNA.**	96.607
5	**PREDICTED: Rattus norvegicus similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (LOC290634), mRNA.**	95.755
6	**PREDICTED: Rattus norvegicus similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (LOC364848), mRNA.**	88.445
7	**Rattus norvegicus phosphoglycerate kinase 1 (Pgk1), mRNA.**	88.077
8	**PREDICTED: Rattus norvegicus similar to glyceraldehyde-3-phosphate dehydrogenase (LOC499433), mRNA.**	88.022
9	**PREDICTED: Rattus norvegicus similar to glyceraldehyde-3-phosphate dehydrogenase (LOC498881), mRNA.**	86.707
10	**PREDICTED: Rattus norvegicus similar to glyceraldehyde-3-phosphate dehydrogenase (LOC498618), mRNA.**	81.792
11	**PREDICTED: Rattus norvegicus similar to glyceraldehyde-3-phosphate dehydrogenase (LOC498099), mRNA.**	81.478
12	**PREDICTED: Rattus norvegicus similar to L-lactate dehydrogenase A chain (LDH-A) (LDH muscle subunit) (LDH-M) (LOC307731), mRNA.**	81.340
13	**Rattus norvegicus monocarboxylate transporter (Slc16a3), mRNA.**	75.782
14	**PREDICTED: Rattus norvegicus similar to glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (EC 1.2.1.12) - mouse (LOC502770), mRNA.**	73.989
15	**Rattus norvegicus beta-glo (MGC72973), mRNA.**	-72.180
16	**PREDICTED: Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase (Gapd), mRNA.**	70.262
17	**PREDICTED: Rattus norvegicus similar to triosephosphate isomerase (LOC500959), mRNA.**	70.109
18	**Rattus norvegicus hydroxysteroid (17-beta) dehydrogenase 1 (Hsd17b1), mRNA.**	69.775
19	**Rattus norvegicus cellular retinoic acid binding protein 2 (Crabp2), mRNA.**	69.463
20	**PREDICTED: Rattus norvegicus triosephosphate isomerase 1 (Tpi1), mRNA.**	68.187
21	**Rattus norvegicus aldolase A (Aldoa), mRNA.**	67.001
22	**Rattus norvegicus glucose phosphate isomerase (Gpi), mRNA.**	66.845
23	**Rattus norvegicus cytochrome P450, family 17, subfamily a, polypeptide 1 (Cyp17a1), mRNA.**	-66.584
24	**Rattus norvegicus BCL2/adenovirus E1B 19 kDa-interacting protein 3 (Bnip3), mRNA.**	65.114
25	**PREDICTED: Rattus norvegicus similar to Tpi1 protein (LOC498731), mRNA.**	61.568
26	**PREDICTED: Rattus norvegicus similar to glyceraldehyde-3-phosphate dehydrogenase (LOC500506), mRNA.**	61.285
27	**Rattus norvegicus macrophage migration inhibitory factor (Mif), mRNA.**	59.309
28	**Rattus norvegicus connective tissue growth factor (Ctgf), mRNA.**	-57.533
29	**PREDICTED: Rattus norvegicus similar to FAD104 (predicted) (LOC294925), mRNA.**	55.358
30	**PREDICTED: Rattus norvegicus 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (Hmgcs1), mRNA.**	55.129
31	**PREDICTED: Rattus norvegicus similar to macrophage migration inhibitory factor (LOC500271), mRNA.**	51.790

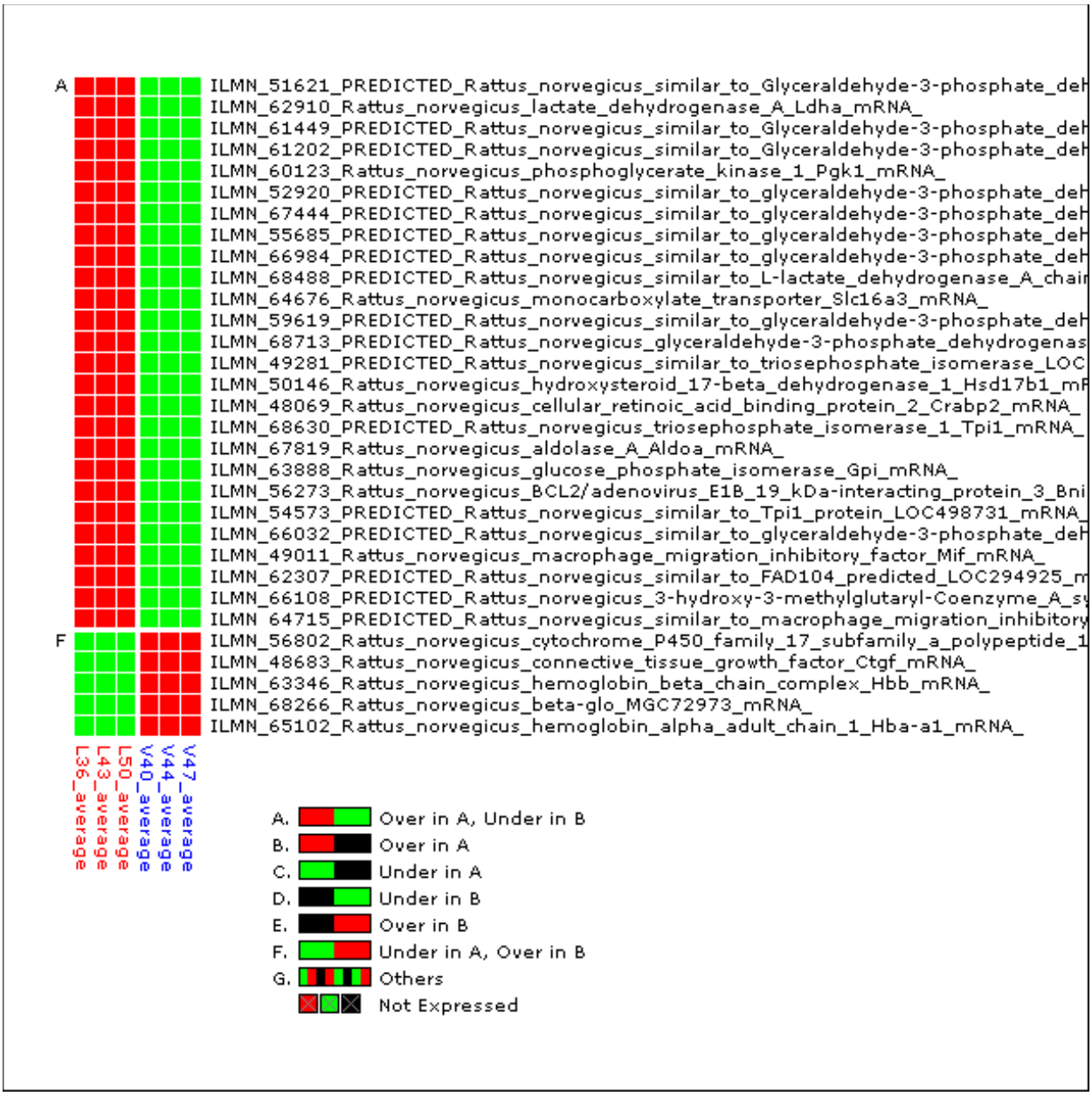


Figure 35: Expression pattern grid for low oxygen group vs. *in vivo* control.

Table 6: Differentially expressed genes in the high oxygen group vs. the *in vivo* control

Rank	Gene	J5 score
1	**Rattus norvegicus hemoglobin alpha, adult chain 1 (Hba-a1), mRNA.**	-92.132
2	**Rattus norvegicus hemoglobin beta chain complex (Hbb), mRNA.**	-89.366
3	**Rattus norvegicus cytochrome P450, family 17, subfamily a, polypeptide 1 (Cyp17a1), mRNA.**	-65.982
4	**Rattus norvegicus matrix Gla protein (Mgp), mRNA.**	-65.381
5	**Rattus norvegicus procollagen, type I, alpha 2 (Col1a2), mRNA.**	-65.208
6	**Rattus norvegicus cellular retinoic acid binding protein 2 (Crabp2), mRNA.**	64.659
7	**PREDICTED: Rattus norvegicus 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (Hmgcs1), mRNA.**	58.792
8	**Rattus norvegicus beta-glo (MGC72973), mRNA.**	-57.056
9	**Rattus norvegicus connective tissue growth factor (Ctgf), mRNA.**	-55.800
10	**Rattus norvegicus lactate dehydrogenase A (Ldha), mRNA.**	53.114
11	**PREDICTED: Rattus norvegicus similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (LOC295452), mRNA.**	52.366
12	**Rattus norvegicus similar to acetyl CoA transferase-like (MGC95138), mRNA.**	51.390
13	**PREDICTED: Rattus norvegicus insulin-like growth factor binding protein 7 (predicted) (Igfbp7_predicted), mRNA.**	-50.872
14	**PREDICTED: Rattus norvegicus similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (LOC290634), mRNA.**	50.305
15	**Rattus norvegicus decorin (Dcn), mRNA.**	-49.927
16	**PREDICTED: Rattus norvegicus similar to ribosomal protein S19 (LOC314733), mRNA.**	-49.395
17	**PREDICTED: Rattus norvegicus procollagen, type VI, alpha 3 (predicted) (Col6a3_predicted), mRNA.**	-49.377
18	**Rattus norvegicus apolipoprotein E (Apoe), mRNA.**	-49.181
19	**PREDICTED: Rattus norvegicus similar to glyceraldehyde-3-phosphate dehydrogenase (LOC498099), mRNA.**	45.900
20	**Rattus norvegicus ribosomal protein S9 (Rps9), mRNA.**	-44.628
21	**Rattus norvegicus lumican (Lum), mRNA.**	-44.346
22	**Rattus norvegicus fucosidase, alpha-L- 1, tissue (Fuca), mRNA.**	43.149
23	**Rattus norvegicus LR8 protein (Lr8), mRNA.**	-41.078
24	**PREDICTED: Rattus norvegicus similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (LOC364848), mRNA.**	41.028
25	**Rattus norvegicus 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (Hmgcr), mRNA.**	40.961
26	**PREDICTED: Rattus norvegicus similar to glyceraldehyde-3-phosphate dehydrogenase (LOC499433), mRNA.**	40.957
27	**Rattus norvegicus seminal vesicle secretion 5 (Svs5), mRNA.**	-39.610
28	**PREDICTED: Rattus norvegicus similar to glyceraldehyde-3-phosphate dehydrogenase (LOC498881), mRNA.**	39.533
Rank	**Gene**	**J5 score**
29	**Rattus norvegicus similar to myo-inositol 1-phosphate synthase A1, mRNA (cDNA clone MGC:93930 IMAGE:7113337), complete**	39.293
30	**PREDICTED: Rattus norvegicus similar to L-lactate dehydrogenase A chain (LDH-A) (LDH muscle subunit) (LDH-M) (LOC307731), mRNA.**	38.778
31	**Rattus norvegicus phosphoglycerate kinase 1 (Pgk1), mRNA.**	38.514
32	**PREDICTED: Rattus norvegicus similar to 40S ribosomal protein S9 (LOC367102), mRNA.**	-37.352
33	**PREDICTED: Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase (Gapd), mRNA.**	37.016
34	**Rattus norvegicus follistatin (Fst), mRNA.**	36.889
35	**PREDICTED: Rattus norvegicus similar to 40S ribosomal protein S19 (LOC363531), mRNA.**	-36.833
36	**Rattus norvegicus hydroxysteroid (17-beta) dehydrogenase 1 (Hsd17b1), mRNA.**	36.764

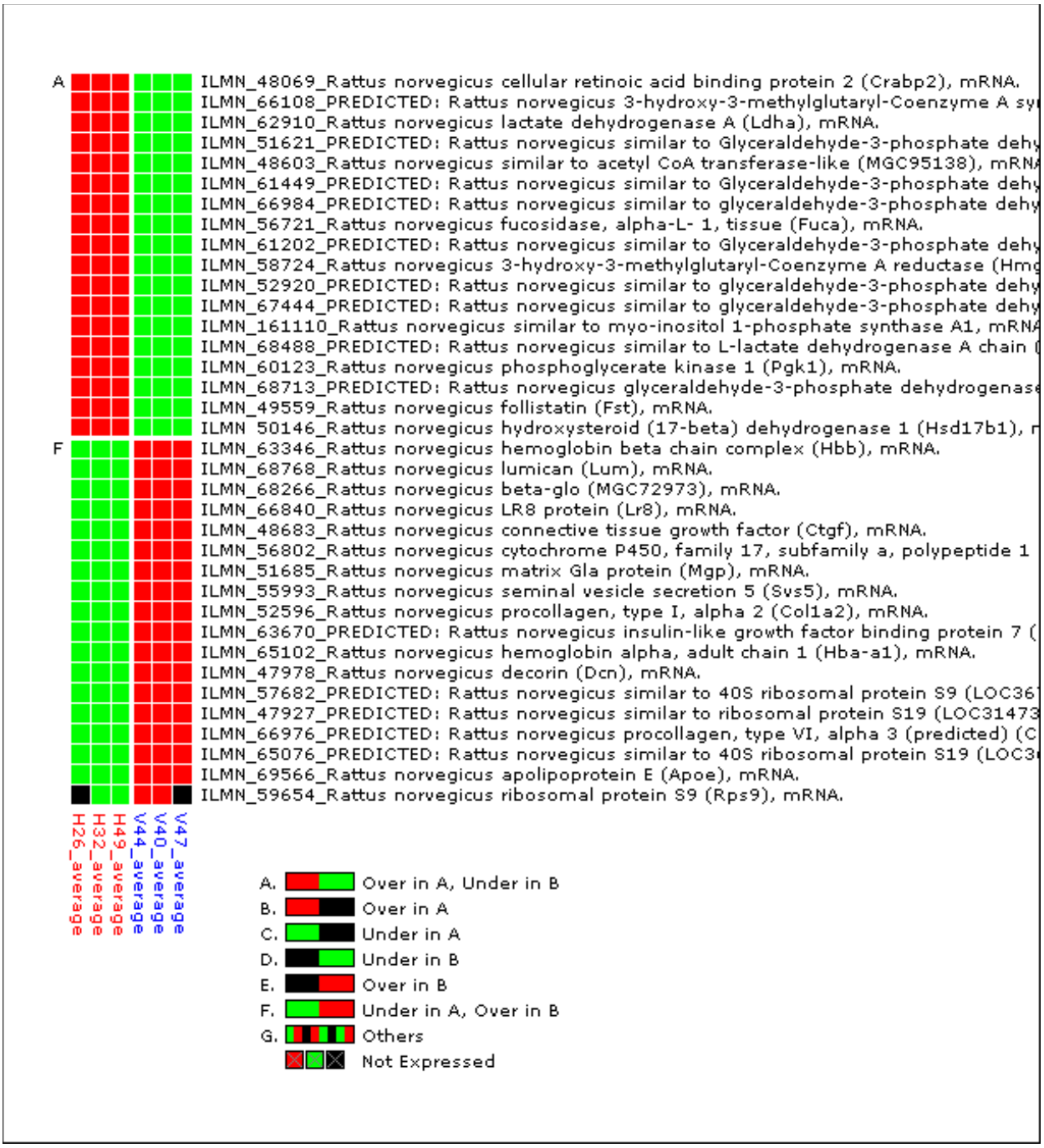


Figure 36: Expression pattern grid for high oxygen group vs. *in vivo* control.

5.3.2.1 Differentiation and Growth

In analyzing the sets differentially expressed genes (Table 5 and 6), it was uncovered that both the high oxygen group and low oxygen group differentially express a gene when compared to the *in vivo* control that plays a role in granulosa cell proliferation and differentiation. In both high and low oxygen groups, connective tissue growth factor is downregulated. During preantral follicle development, this gene is abundantly expressed in rat granulosa cells[119]. Due to the expression patterns of connective tissue growth factor throughout follicular development in the rat, it was found to play a role in follicle growth and thecal cell recruitment [119]. However, *in vitro*, connective tissue growth factor has been found to be downregulated when cultured with FSH [119]. For this reason, this particular growth factor showed similar expression levels in both the high and low oxygen groups, but instead is altered due to the presence of FSH in both culture groups.

Although connective tissue growth factor remained the only growth and differentiation factor differentially expressed in the low oxygen group, it was discovered that the high oxygen group differentially expressed two additional genes that help regulate this process, follistatin, and decorin.

Follistatin was found to bind activin, reducing activin activity [123]. Activin is produced by the granulosa cells and, in an autocrine fashion, regulates the differentiation of granulosa cells [123, 124]. In the case of the undifferentiated granulosa cells present in preantral follicles, activin induces FSH receptors, allowing for the follicles to become responsive to FSH. FSH, in turn, stimulates the production of additional activin and activin receptors in the granulosa cells to further increase FSH receptor expression. It is believed that along with this effect on FSH

receptors, activin promotes estradiol production, leading to not only the differentiation of the granulosa cells, but also the prevention of luteinization and atresia [123].

Follistatin has been shown to have the opposite effect *in vitro*. It inhibits the effects of activin, leading to the premature luteinization of the preantral follicle or atresia [123]. In this study, follistatin was found to have been upregulated in the high oxygen group compared to the control, but not differentially expressed in the low oxygen group when compared to the control.

Decorin is a small proteoglycan that binds transforming growth factor-beta (TGF-beta) inhibiting TGF-beta activity in the ovary [125]. Decorin helps regulate cell growth within preantral follicles by participating in a negative feedback system to control TGF-beta activity [125]. Contrary to follistatin, decorin was downregulated in the high oxygen group compared to the control. This may result in an abnormally high level of TGF-beta activity within these preantral follicles. Decorin was not found to be differentially expressed in the low oxygen group when compared to the control.

5.3.2.2 Steroidogenesis

In both the high and low oxygen groups, cytochrome p450 family 17 subfamily A polypeptide 1 (Cyp17A1) was downregulated compared to the *in vivo* control group. The Cyp17A1 gene is expressed by the theca cells and encodes for the enzyme steroid 17-alpha-hydroxylase [121]. This enzyme catalyzes the hydroxylation of progesterone or pregnenolone along with the cleavage of a two-carbon side chain resulting in the formation of androstenedione or dehydroepiandrosterone. As a result, this is a critical enzyme that properly maintains the androgen supply that is utilized for estrogen synthesis [121]. By downregulating the expression of this gene, estrogen levels could be reduced, limiting the availability of estrogen to the developing follicles.

In addition to Cyp17A1, it was discovered that two other genes responsible for regulating steroidogenesis, apolipoprotein-E and insulin-like growth factor binding protein 7 were also downregulated in follicles in the high oxygen group, but not in follicles from the low oxygen group.

Similar to Cyp17A1, apolipoprotein-E is also expressed by the theca cells. This protein then acts in an autocrine manner in order to regulate androgen production within the theca cells [126].

Insulin-like growth factor binding protein 7 is expressed in the granulosa cells. Although the role of insulin-like growth factor binding protein 7 has not been elucidated in regards to preantral follicle development, it has been found to negatively control the steroidogenesis of granulosa cells in more mature follicles [120]. By functioning in this capacity, it is believed that this protein serves to sustain proper steroid levels within the follicle.

5.3.2.3 Carbohydrate Metabolism

Carbohydrate metabolism in ovarian follicles is consistent with cells possessing adequate proliferative potential [127]. Granulosa cells in every stage of follicle development produce lactate as the major product of follicular carbohydrate metabolism [128]. It has been theorized particularly for preantral follicles without a vascular supply that the majority of oxygen is consumed in the outer cells of the follicle and that little if any oxygen actually diffuses to the interior region of the follicular unit [129]. Therefore, this would create an anaerobic environment and drive the formation of lactate. However, it has been discovered that monolayers of granulosa cells cultured in an aerobic environment still produced significant quantities of lactate [130]. As a result, it is believed that these cells produce the lactate because

it is the most efficient method for providing the considerable quantity of ATP necessary for rapid growth rates [129].

With the significant concentration of FSH facilitating granulosa cell proliferation present in both the high and low oxygen culture conditions, it was not surprising that several genes involved in carbohydrate metabolic pathways, such as lactate dehydrogenase A and glyceraldehydes-3-phosphate dehydrogenase, were found to be upregulated in both the high and low oxygen groups. By administering a relatively high concentration of FSH compared to the level experienced by preantral follicles *in vivo*, the granulosa cells in the cultured preantral follicles in both the high and low oxygen group proliferated at a more rapid rate, increasing the cell's ATP requirements. As a result, the glycolysis rate increased, driving the expression levels of genes that participate in carbohydrate metabolism higher.

5.4 CONCLUSIONS

At the onset of this chapter, it was suggested that the low oxygen group would have a gene expression pattern that more closely resembled the gene expression levels for the *in vivo* control than the high oxygen group. This hypothesis was based on the results from Chapter 4 that showed a significant increase in oocyte viability and maturation when follicles were cultured in a dynamic oxygen environment than if they were cultured in an ambient oxygen environment. Although, it was also recognized that when compared to the *in vivo* control oocytes from Chapter 4, there was a significant decrease in oocyte viability and maturation for both the dynamic oxygen group and the ambient oxygen group.

As expected, several genes in both the high and low oxygen groups were found to be differentially expressed when compared to the *in vivo* control. However, when the correlation plots for the low oxygen group vs. the *in vivo* control and the high oxygen group vs. the *in vivo* control were compared, it was found that the low oxygen group displayed a better correlation to the *in vivo* control than the high oxygen group. The difference between the R^2 values was small, but it was consistent, remaining robust to several different normalization methods. This supports the conclusion that the difference is due to biological variations.

By analyzing the individual genes that were differentially expressed in both culture groups compared to the control, it was uncovered that both the low oxygen group and the high oxygen group differentially express genes that control three crucial processes that participate in the regulation of follicle development, the differentiation and growth of granulosa cells, steroidogenesis, and carbohydrate metabolism. However, in addition to the two shared genes that were differentially expressed in both culture groups, connective tissue growth factor and Cyp17A1, the high oxygen group differentially expressed another four genes that had roles in the proper differentiation and growth of granulosa cells and the maintenance of appropriate levels of steroidogenesis, follistatin, decorin, apolipoprotein-E, and insulin-like growth factor binding protein 7. These additional genes could possibly lead to even further desynchronized preantral follicle development.

6.0 SUMMARY, IMPLICATIONS, AND FUTURE DIRECTIONS

6.1 SUMMARY OF MAIN FINDINGS

The main findings for the entire study are divided below by the specific research aims. The subsequent implications of these findings are presented in the following section 6.2.

6.1.1 Maintenance and Support of Follicle Structural Integrity

Previous studies showed that follicles larger than those of a mouse lose three-dimensional integrity when cultured on a flat surface. We hypothesized that, by culturing follicles in a suspension culture system, the three-dimensional integrity of the follicular unit would be better supported. Furthermore, the utilization of alginate microcapsules could help maintain proper follicle morphology. In this aim, we studied the effect that structural support, nutrient delivery, and mechanical shear stress may have on follicular growth and morphology. We demonstrated that suspension culture in combination with hydrogel microencapsulation maintains follicular growth and morphology more effectively than conventional culture in tissue culture wells. Follicle flattening is eliminated in both suspension culture systems. In contrast to static culture, follicles are uniformly supported during suspension culture, unable to adhere to a surface, and are protected from shear stress when embedded in calcium alginate microcapsules. In addition, it

was discovered that in order for encapsulated follicles to maintain the growth rate of unencapsulated follicles, the inclusion of FSH into the scaffold is required.

6.1.2 Development of a Novel Dynamic Oxygen Environment

It is likely that *in vivo* follicles experience a transition from relative hypoxia in early development to the high volume of blood flow and oxygen delivery for pre-ovulatory follicles. We hypothesized that the difficulty in achieving *in vitro* oocyte maturation may be due to the dysregulation of follicle development by exposure of early stage follicles to inappropriate oxygen concentrations. Therefore, in this aim, we studied the effect that a dynamic oxygen delivery protocol that more closely mimicked the oxygen environment of the native ovary would have on oocyte viability and maturation. We found that the dynamic oxygen environment produced a significantly higher yield of healthy oocytes than the static control. More importantly, the oocytes from the dynamic oxygen environment were significantly more efficient at progressing through germinal vesicle breakdown than their compliments in the ambient oxygen group. Furthermore, the dynamic oxygen group was able to produce oocytes that were capable of undergoing parthenogenetic activation and fertilization.

6.1.3 Preantral Follicle Gene Expression Analysis

After observing the advantages of culturing follicles in the dynamic oxygen environment as compared to the ambient oxygen environment, we hypothesized that by placing the follicles in the ambient oxygen group in an environment more suitable to mature graafian follicles, the granulosa cells may not differentiate properly, leading to desynchronized follicle development.

In this aim, we studied the effect that the oxygen environment had on preantral follicle gene expression. We discovered that follicles cultured in a low oxygen environment have an expression pattern that more closely resembles the expression pattern from *in vivo* control follicles when compared with follicles cultured in a high oxygen environment. Furthermore, it was found that in addition to the two shared genes that were differentially expressed in both culture groups, connective tissue growth factor and Cyp17A1, the high oxygen group differentially expressed another four genes that had roles in the proper differentiation and growth of granulosa cells and the maintenance of appropriate levels of steroidogenesis, follistatin, decorin, apolipoprotein-E, and insulin-like growth factor binding protein 7.

6.2 IMPLICATIONS OF MAIN FINDINGS

6.2.1 Follicle Culture System Development

The findings in all three aims of this study contribute additional knowledge and scientific understanding to the field of *in vitro* ovarian follicle development, predominantly the development of preantral follicles. The suspension culture and microencapsulation findings indicate that the traditional culture of follicles on a flat surface will not adequately support whole follicle development for these larger follicles. When culturing these rapidly growing follicular units on foreign surfaces with no three dimensional support, follicles will plate down to the surface and disrupt proper structural integrity. It stresses the need to consider three-dimensional support in future culture systems.

The findings from the oxygen tension study reveal the importance of the unique oxygen environment experienced by these follicles in the native ovary. As the field continues to develop culture systems to support larger follicles, the delivery of appropriate levels of nutrients such as oxygen will become more important. These findings can be optimized for future culture systems in order to deliver the appropriate oxygen levels throughout the maturation of larger follicles. This will serve to increase efficiency of producing viable and mature oocytes.

The results from the gene expression analysis study could have an impact on the growth rate of cultured follicles. One of the most notable findings in this study was the drastic increase in carbohydrate metabolism due to the relatively high concentrations of FSH in the culture media. This suggests that the preantral follicles in culture progressed much more rapidly than the *in vivo* control follicles. Therefore, it is possible that preantral follicle development may be better maintained by culturing preantral follicles at low oxygen and FSH concentrations for longer periods of time. By slowing down the growth of the follicles, they may be able to better manage their basement membrane integrity and the maturation of the oocyte.

6.2.2 Fertility Restoration and the Derivation of Embryonic Stem Cell Lines

As stated in the initial chapter of this study, the overall goal is to develop a culture system that has the ability to produce mature oocytes in order to restore fertility and prove that banked ovarian tissue could serve as a source for embryonic stem cells. By demonstrating successful fertilizations and parthenogenetic activations, the findings from the oxygen tension study imply that the dynamic oxygen culture system is a viable system for both the restoration of fertility and the derivation of embryonic stem cell lines.

Although the culture system will have to be scaled up to support the needs of the larger primate follicles, the concepts discovered in this study will serve as a foundation for proper *in vitro* follicle development.

6.3 FUTURE DIRECTIONS

The findings within this study have contributed to the development of a culture system capable of producing mature oocytes. This will provide the basis for future studies involving the production of embryos and the derivation of stem cell lines. Although this study has demonstrated proof of principal through the fertilization of these oocytes, the intracytoplasmic sperm injection process and subsequent embryo culture must be optimized. Once the process of creating and culturing embryos becomes more efficient, the research can progress to the production of live offspring and the derivation of embryonic stem cell lines. Therefore, the future direction of this research is outlined in the following aim.

Future Aim One: Create embryos from *in vitro* derived oocytes.

In this aim**,** we will continue to develop the enabling technology of *in vitro* folliculogenesis to the natural end point of embryo derivation. We will address three hurdles limiting the efficiency of this process.

6.3.1 Future Aim 1.1: Develop intracytoplasmic sperm injection (ICSI) techniques to overcome the problem of premature Zona Pellucida hardening in *in vitro* cultured oocytes.

By performing ICSI on ovulated oocytes, we will overcome the zona hardening problem encountered in most oocyte culture systems. We have already performed trial studies of this technique with our *in vitro* derived oocytes. Micromanipulation has shown that the zona of our oocytes is indeed hardened but is easily broached by the peizo drill. Rat sperm heads can also be obtained by cutting with the peizo instrument and then placed into the cultured oocytes.

Alternately there is another technique we can use to treat Zona hardening. Fetuin at concentrations ranging from 0.1 to 10μg/mL has been shown to reduce zona hardening [131]. These same investigators have reported some success in achieving fertilization of *in vitro* matured oocytes incubated with fetuin prior to insemination [131].

6.3.2 Future Aim 1.2: Do embryos from *in vitro* derived oocytes have alterations in gene regulation?

In order to ascertain whether oocytes derived from culture have any alteration that may affect embryo quality but not be readily apparent morphologically, we will perform DNA profiling of two-cell and blastocyst stage embryos derived in three different ways. We will obtain eggs derived *in vivo*, by dissection of oviducts of animals subject to routine superovulation. These *in vivo* derived eggs will be fertilized either by routine IVF (insemination by placing activated sperm in the same culture dish with the ovulated eggs) or by ICSI (intracytoplasmic sperm injection). Cultured follicles will be grown, ovulated and matured to the metaphase II stage as

described in Aim 1.1 above and in preliminary data section. Cultured oocytes will be fertilized by ICSI.

6.3.3 Future Aim 1.3: Enhance the efficiency of *in vitro*-induced oocyte ovulation.

Follicle stimulating hormone (FSH) and leutenizing hormone (LH) are hormones that are secreted into the blood from the anterior pituitary gland. Rat Follicles have traditionally been grown in the absence of LH and in static FSH concentrations that are several orders of magnitude above normal serum concentrations. However, preantral follicles exist in the avascular cortex of the ovary. The FSH available to these small follicles would have to diffuse from nearby antral follicles that do have a blood supply. Furthermore, FSH and LH serum levels fluctuate with the different days of the reproductive cycle. Therefore, over the course of development of a follicle from primordial to matured graafian follicle, the follicle is subjected to an increasing range of FSH and LH concentrations. Initially, concentrations are low but as vascular supply develops around the follicle, it is exposed to the peaks and troughs of FSH and LH fluctuation in the serum. By excluding LH and imposing a static FSH concentration on cultured follicles, conventional culture fails to recreate the dynamic FSH environment present throughout the *in vivo* follicle development process. We know that granulosa cells are extremely sensitive to FSH concentrations. It is likely that granulosa cells in follicles also respond to variation in gonadotopin levels very differently than constant dosing. In our preliminary studies we have determined that we have improved survival and growth of follicles in long term culture when fresh FSH is added to the cultures every four days in increasingly higher doses. The studies in this section with expand on these studies to see if pulsatile FSH delivery that mimics the *in vivo* environment and LH dosing improves the ovulation rate of cultured follicles.

6.3.4 Future Aim 1.4: Determine the effect of EGF on the *in vitro* maturation of rat oocytes.

Our preliminary studies have shown that the oocytes cultured in the suspension culture system are able to resume meiosis and progress to metaphase II. However, the *in vitro* maturation of oocytes to metaphase II must become more efficient for this system to be considered a viable solution. One approach to optimize the *in vitro* maturation of these oocytes is the addition of EGF to the media at the end of the culture.

APPENDIX A

[PATHWAY-EXPRESS ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES]

The goal was to determine which pathways are impacted by the differentially expressed genes. We implemented a pathway level Impact Analysis which was designed to provide both statistical and biological significance in the indication of which pathways may be affected by the observed changes in gene expression. The results are summarized as Impact scores and p-values in Table 7. Pathway-Express orders the affected pathways in the decreasing order of their expected importance for the given condition, renders KEGG pathway diagrams indicating impacted components (Figures 37-41). There were no pathways found to be significantly impacted by the differentially expressed genes from the low oxygen group. Five pathways were found to be significantly impacted by the differentially expressed genes from the high oxygen group shown in figures 37-41.

Table 7: Pathways found to be impacted by the differentially expressed genes from the high oxygen group

Rank	Pathway Name	Impact Factor	#Genes in Pathway	#Input Genes in Pathway	#Pathway Genes on Chip	%InputGenes in Pathway	%Pathway Genes in Input	p-value	corrected p-value	gamma p-value	corrected gamma p-value
1	TGF-beta signaling pathway	9.132	64	2	64	8.333	3.125	0.001561	0.001561	0.001096	0.001096
2	ECM-receptor interaction	8.81	62	2	62	8.333	3.226	0.001466	0.001466	0.001464	0.001464
3	Focal adhesion	5.78	163	2	163	8.333	1.227	0.009676	0.009676	0.020941	0.020941
4	Alzheimer"s disease	4.896	22	1	22	4.167	4.545	0.019937	0.019937	0.044081	0.044081
5	Neurodegenerative Disorders	4.73	26	1	26	4.167	3.846	0.023521	0.023521	0.050576	0.050576

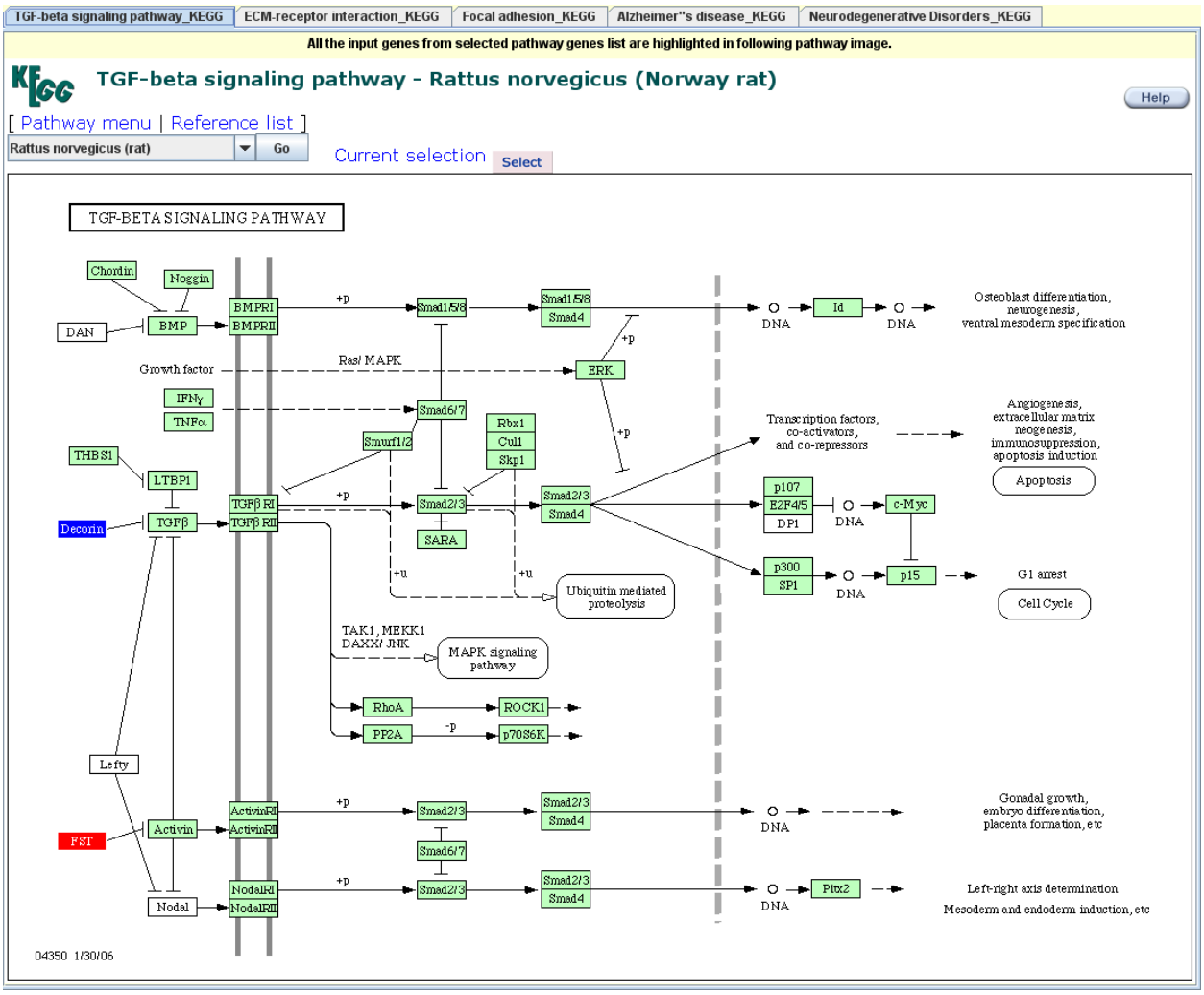


Figure 37: TGF-beta pathway.

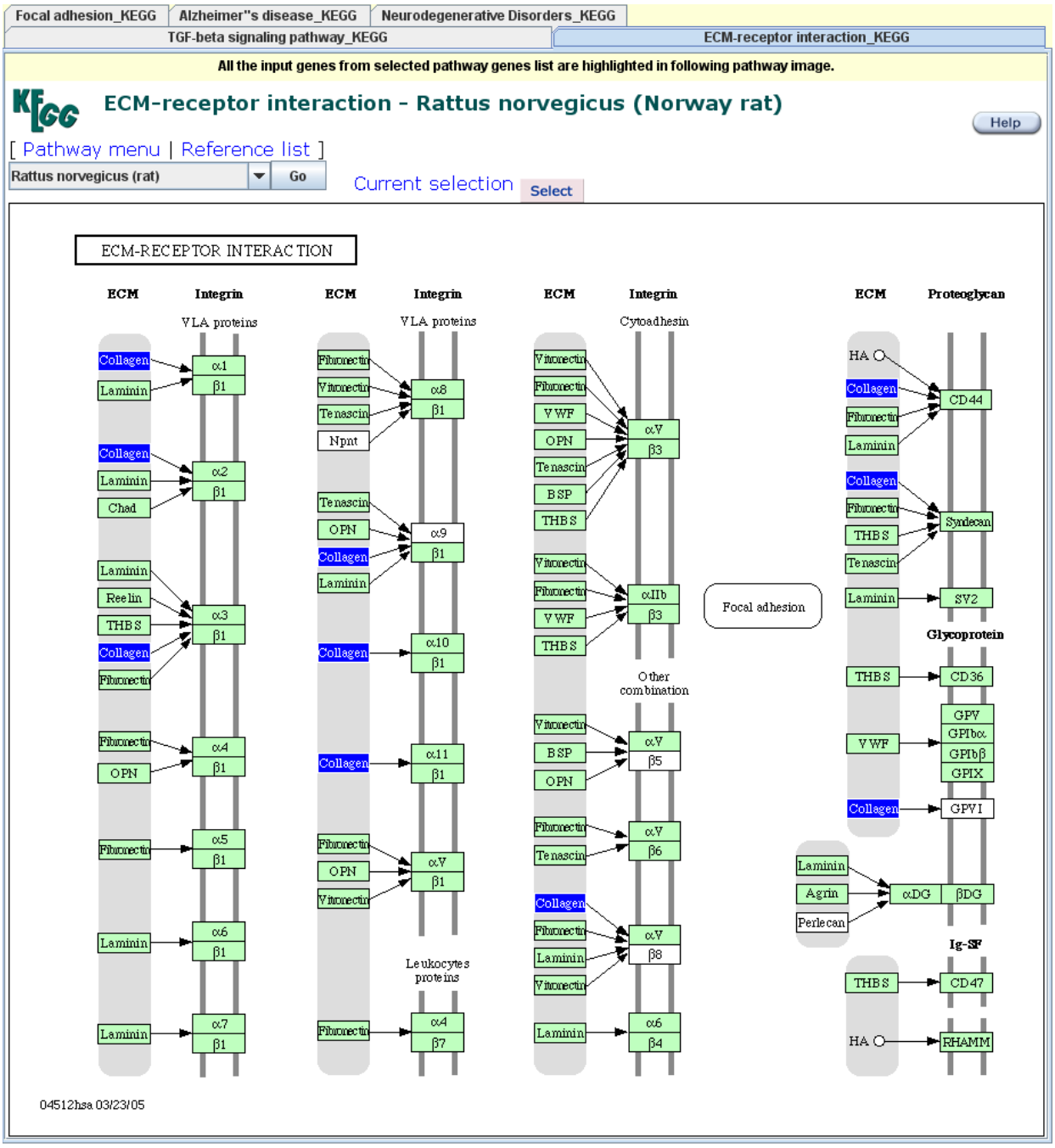


Figure 38: ECM-receptor pathway.

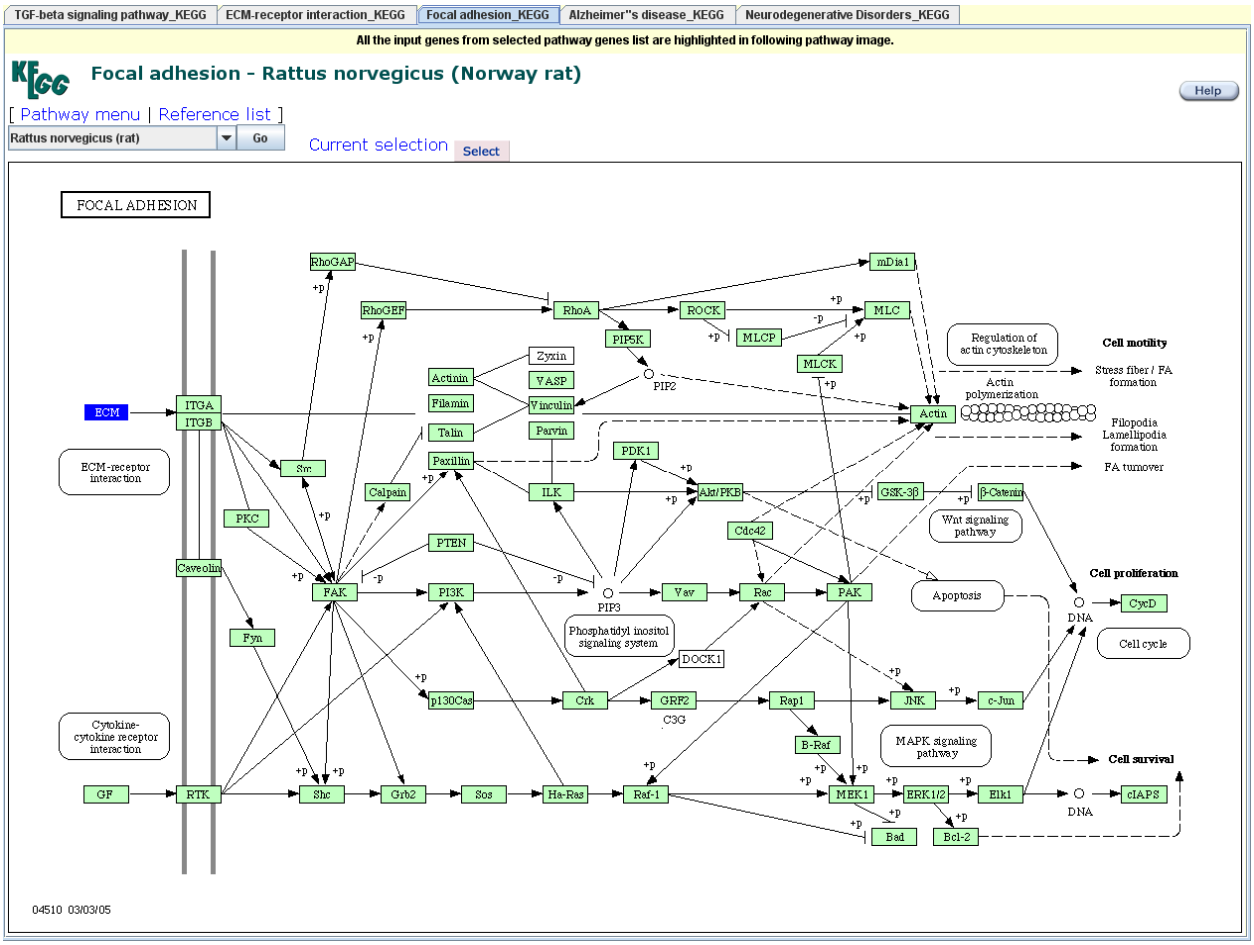


Figure 39: Focal Adhesion pathway.

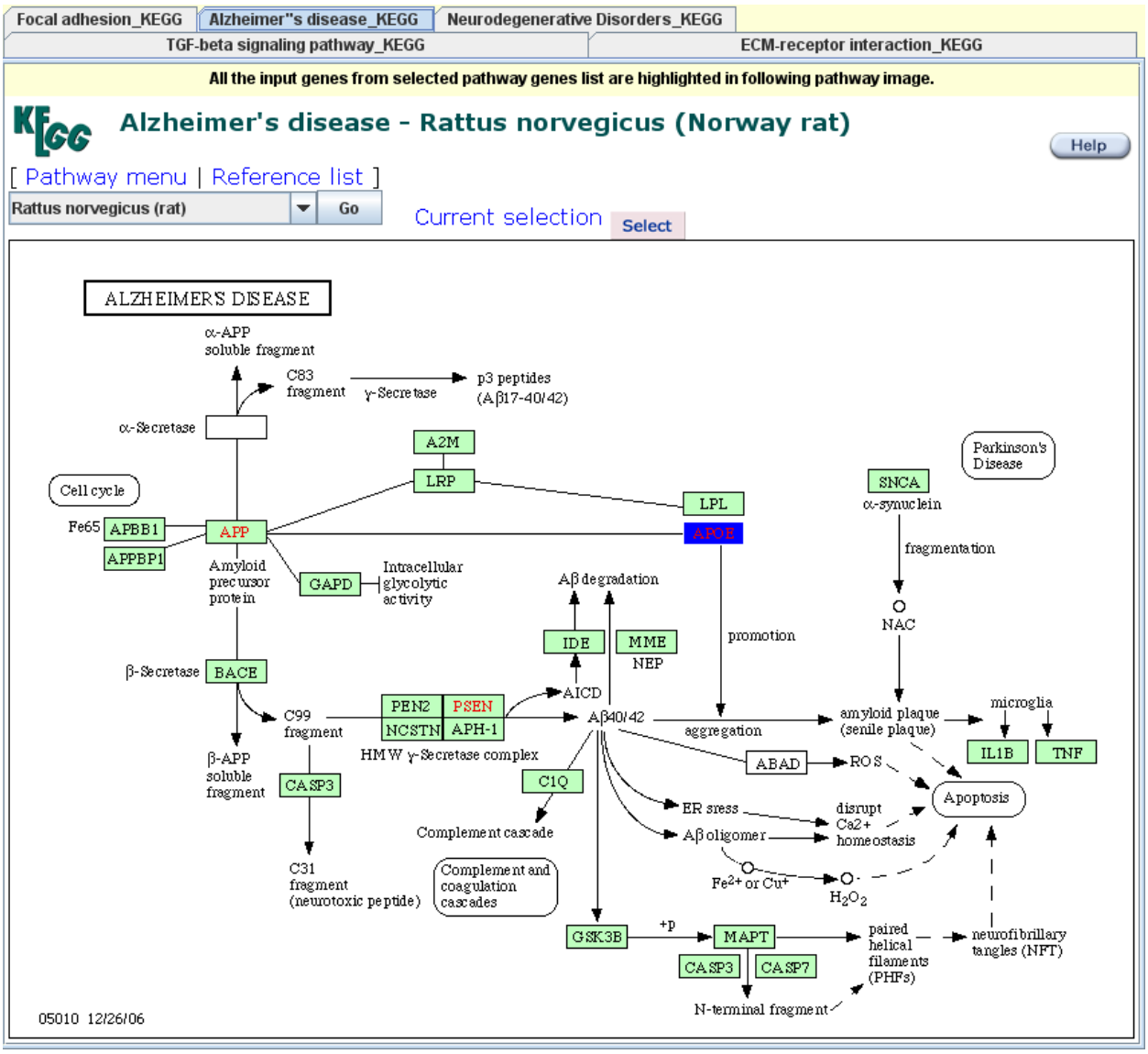


Figure 40: Alzheimer's disease pathway.

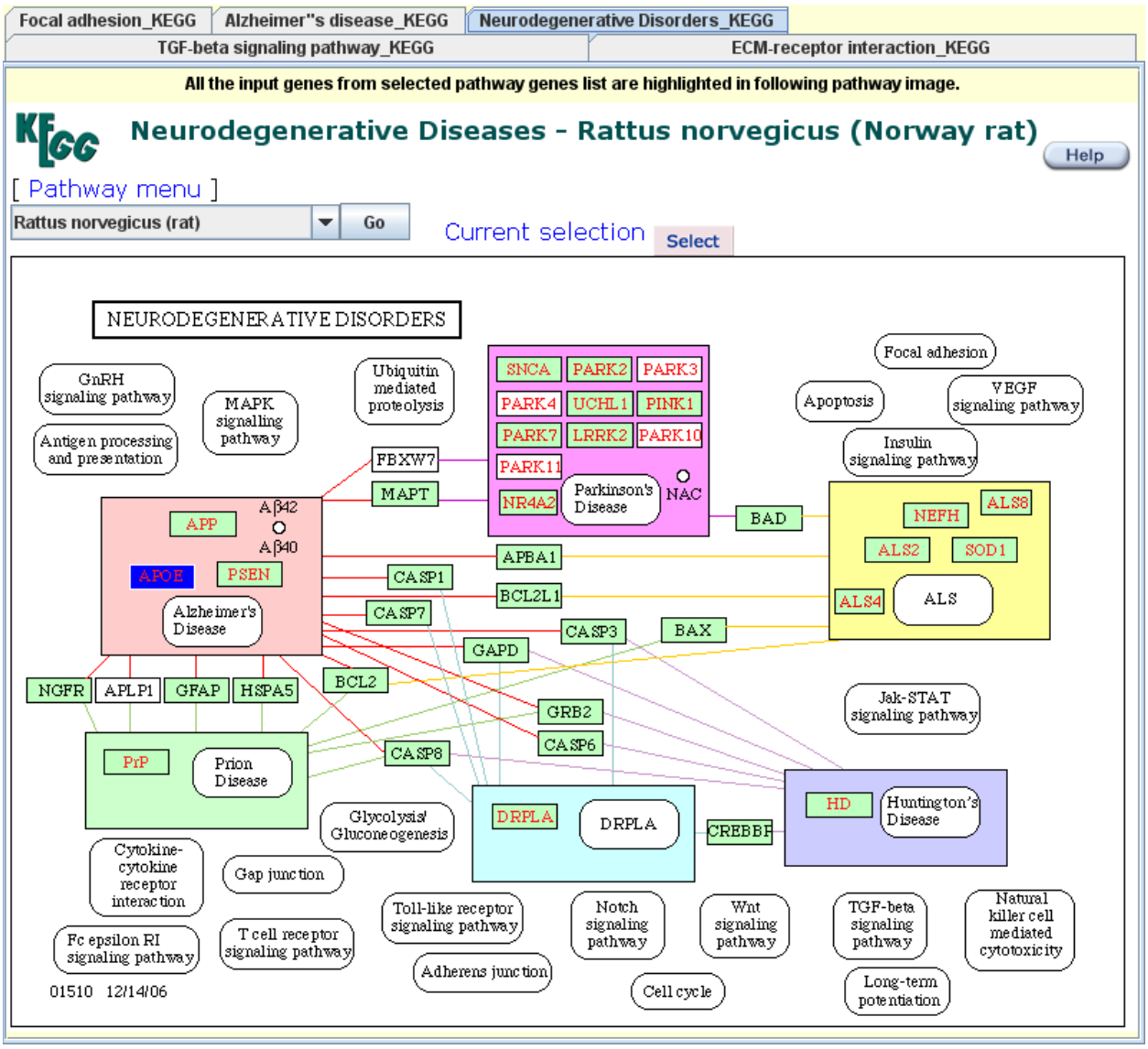


Figure 41: Neurodegenerative disorders pathway.

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