TOWARDS THE DEVELOPMENT OF AN ARTIFICIAL OVARY

by

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ABSTRACT

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Ovarian preantral follicles often flatten and rupture during static culture in a tissue culture well. In this study, growth and morphology were evaluated for rat and mouse preantral follicles cultured for 72 hours in conventional culture wells and in suspension culture systems, consisting of orbiting test tubes and rotating-wall vessels. Follicles cultured in the orbiting test tubes had increased growth rates relative to the rate in the conventional culture wells, and experienced neither flattening nor rupture. The majority of follicles cultured in the rotating-wall vessel appeared to experience shear damage. In order to provide a potential barrier from shear stress during suspension culture, follicles were encapsulated in calcium alginate gels and growth and morphology were evaluated in the conventional and suspension culture systems. Encapsulated follicles more closely resembled an in vivo morphology and did not flatten nor rupture. Our studies suggest that suspension culturing in a rotating-wall vessel in combination with microencapsulation supports more natural three-dimensional follicular growth and morphology. The approach described herein is the first step toward the development of an artificial ovary. Additionally, this new culture system maintains follicles in a more natural morphology and will provide an important new avenue for further detailed investigation of the complex regulation of ovarian follicle development.

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1.0 INTRODUCTION

1.1 Fertility Preservation

Female patients undergoing aggressive radiotherapy or chemotherapy to treat cancer are often sacrificing their human right to procreate since these treatments are also responsible for the depletion of ovarian germ cells, or eggs. Females are born with all the eggs they will ever have and once depleted, the ovaries are sterile. In addition, the loss of the follicular unit surrounding the egg results in the inability to produce the hormone estrogen. Although these cancer therapies provide greater than 90 % cure for many cancers in young females [(1)], infertility or severe subfertility is a side effect which impacts the patient's quality of life. When the ovaries are sterile, adult and adolescent women reach premature menopause and children fail to undergo hormonal development for puberty. Therefore, due to the increasing survival success of cancer treatments, there is a great demand for preserving the fertility of young patients prior to or during their reproductive years [(2)].

Studies on fertility after treatment with radiotherapy or chemotherapy have shown that a range of ovarian damage occurs and is dependent on the dosage and duration of treatment and the patient's age, with greater risk in older women [(3)]. The ovaries of many prepubescent females are subjected to high doses of radiation for treatments of cervical, rectal, and central nervous system cancers as well as for Hodgkin's disease in the pelvic lymph nodes [(3)]. A radiation dosage of 4 Gy is the estimated amount that destroys half of the egg supply [(4)], and 20

Gy is the estimated dose causing permanent ovarian failure for women less than 40 years of age [5]. A study on ovarian function in childhood and adolescence demonstrated that six out of eight women treated with total body irradiation obtained ovarian failure [6]. The adverse effects of chemotherapy on ovarian function do not seem to be as severe as with radiotherapy. However, a study on the effect of chemotherapy on ovarian function in 168 young cancer patients demonstrated a 34 % ovarian failure rate with alkylating agents imposing the greatest risk, when compared to other classes of chemotherapeutic agents [3].

As a result, for girls and young women facing potential infertility due to their cancer treatment, a technique that would allow them to bear their own children would be welcome indeed. Recently, ovarian tissue cryopreservation [1,7], or removing and freezing portions of ovaries from girls and women undergoing potentially sterilizing treatments, has begun in the United States and in Europe. Although cryopreservation is an attractive solution, it is an incomplete one. There is currently no reliable technology that can subsequently produce fertile eggs upon thawing of this stored tissue [7,8].

2.0 BACKGROUND AND LITERATURE REVIEW

2.1 Ovarian Follicle Growth and Development

Eggs, or oocytes, are located in ovarian follicles, the main structural and functional unit of the ovary. Healthy follicles provide an appropriate environment for oocyte growth and development and produce the hormones necessary for the development of secondary sexual characteristics and pregnancy [9,10]. As shown in Figure 1, in addition to the egg, which is innermost, an ovarian follicle consists of epithelial-like granulosa cells, which is surrounded by a basement membrane, and mesenchymal theca cells that surrounds the exterior side of the basement membrane [11]. In humans, follicle formation begins during mid-gestation (20 weeks), in which there are about 6 to 7 million oocytes, and lasts to just after birth where there are about 300,000 to 400,000 follicles and will generally remain dormant in a resting pool [10]. It is believed that the remaining oocytes that do not become enclosed within follicles most likely undergo apoptosis [10].

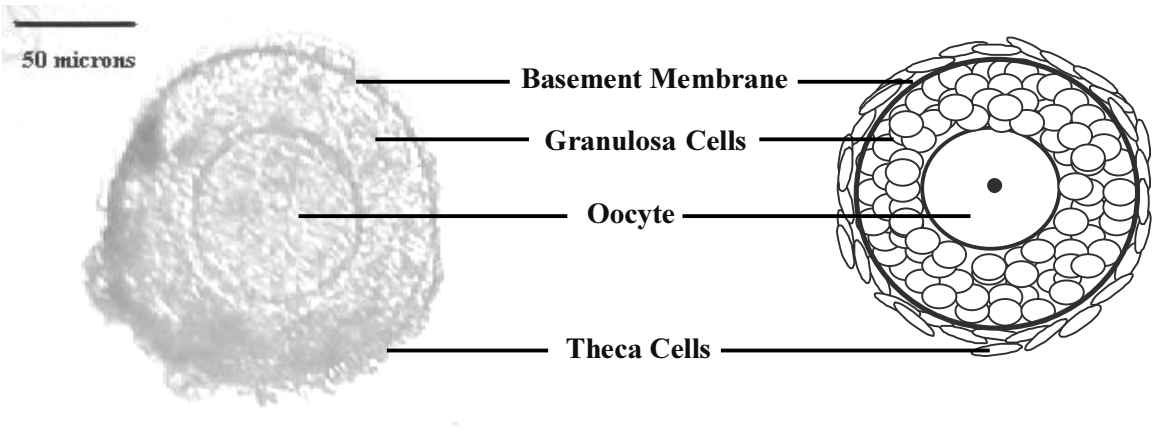

Figure 1 Anatomy of an Ovarian Follicle

An unknown mechanism, called initial recruitment [10], activates a group of the dormant follicles for growth, as shown in Figure 2. This continuous process begins after follicle formation and occurs until the resting pool of follicles is depleted and menopause ensues. When recruited from the resting pool, follicles spend most of their time developing individually through morphologically distinct stages known in order as, primordial, primary, and secondary stages [10,12]. Prior to puberty, secondary follicles develop up to the antral stage, but then undergo apoptosis [12].

From the onset of puberty and during every reproductive cycle, where there are approximately 200,000 follicles present in the human ovary, a cohort of these follicles is recruited from the antral stage for further growth, as shown in Figure 2 [10]. This cyclic recruitment rescues approximately 10 antral follicles (2-5 mm in diameter in humans, 0.2-0.4 mm in diameter in rodents) [10] from apoptosis and is due to the increase in pituitary Follicle Stimulating Hormone (FSH) that circulates during each reproductive cycle (28 days in humans, 4-5 days in rodents). By selection and dominance, one follicle out of the cohort develops into a pre-ovulatory, or Graafian follicle [10]. The reason why one follicle proceeds to the Graafian stage is unknown, however it is believed that this dominant follicle may be more sensitive to FSH [13]. It is known that the dominant follicle grows faster than the others [10] and produces higher levels of estrogens and inhibins, which suppresses the release of FSH, depriving the less developed follicles in the cohort with enough FSH to further their development [14]. As a result, the remaining follicles undergo apoptosis while the Graafian follicle ovulates, releasing its oocyte for fertilization.

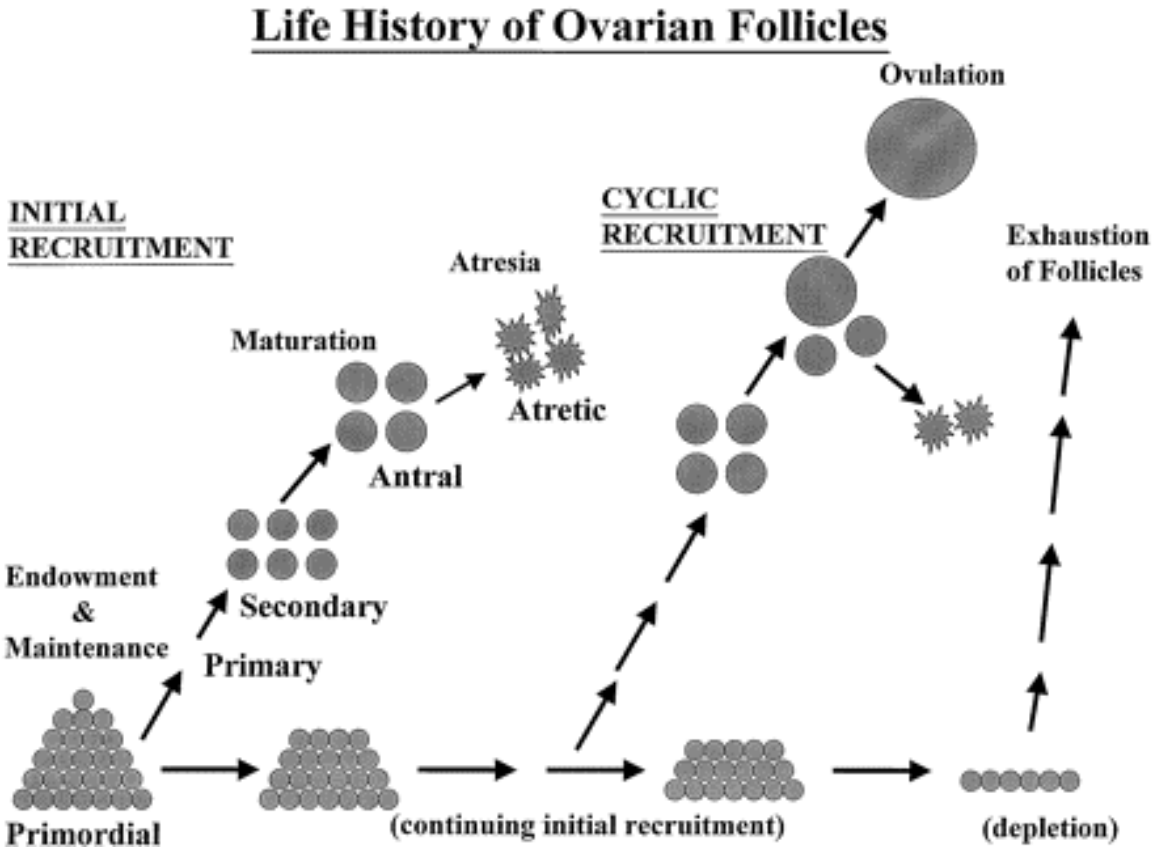

Figure 2 Life History of Ovarian Follicles

(10)

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, or preantral follicles. Their slow growth rate and small size makes them more difficult to isolate for culture [11,15]. Maintaining optimal growth and morphology in vitro is complicated due to the complex follicle structure and tightly controlled dynamics of follicle maturation. Interestingly, follicle flattening and rupture often occurs during culture in a tissue culture well [16,17,18,19]. Follicle rupture is characterized by the disruption of the follicle and the movement of the 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. Therefore, maintaining preantral follicle growth and morphology during *in vitro* culture is the issue we addressed in this study.

2.2 Overview of In Vitro Follicle Culture

In order to determine how preantral ovarian follicles mature, researchers in this field typically monitor follicle growth and morphology during *in vitro* culture. Numerous techniques that vary in media recipes, incubation time, follicle size, and animal model are applied to investigate follicle metabolism during specific growth stages. Currently, the most success of *in vitro* maturation has been with the mouse model. In one study, fertile oocytes were developed in culture from primordial follicles within whole ovaries of neonatal mice [20]. One offspring was produced, but aged prematurely and did not survive very long [21]. Another study has shown that culture of intact preantral mouse follicles can be matured to a fertile stage [22].

Although there has been success with the mouse model, similar success with species that have larger follicles has not been reported. Follicle structure is similar in rats, cattle, pigs, and humans, however the rate of follicle development varies between species [10]. As a result, there is a great difference in the diameter of matured oocytes and follicles [18] and also the granulosa cell number [23]. In addition, more than one follicle can be selected for ovulation in rodents, and pigs [10], while generally only one follicle dominates in the human and bovine system [23]. Therefore, due to such variation between species, a successful culture method with one animal model may not necessarily lead to success with another, but may be possible with the appropriate adjustments.

2.3 Follicle Culture Techniques

2.3.1 Follicle Isolation

Follicles are isolated from the ovary by enzymatic treatment, manual dissection, or a combination of the two. Enzymatic dissociation of small follicles from an ovary is typically performed using collagenase with DNase and is most feasible with small mammalian ovaries. Isolating follicles with enzyme action is achievable with samples of fetal or young ovaries that have soft tissue and not achievable with the more fibrous tissue found in adult human ovaries or other ovaries that have more dense tissue [18]. It is possible to obtain around 140 follicles per ovarian biopsy when exposed to the enzyme for 1 hour at 37 ^{0}C and then for 36 hours at 4 ^{0}C

unless the tissue is dense [24]. Although a large number of small follicles may be obtained with enzyme dissociation, the follicles are isolated without the theca layer, and the basement membrane usually becomes damaged [18]. The basement membrane and theca are necessary for providing structural support to a follicle. The theca influences the formation of an antrum [25,26] and also has biochemical interactions with granulosa cells to promote oocyte development [27,28]. Even when enzyme dissociated follicles are cultured in Matrigel, or basement membrane material, they do not seem to maintain their spherical structure and the effects of the enzyme on cell surface receptors and other molecules are unknown [18].

As a result, manual dissection is a more reliable method. This technique usually involves removing follicles from each other and from the ovary using needles on syringes and under a dissecting microscope. The disadvantages of manual dissection are that it is time consuming and relatively difficult, thus a smaller amount of follicles can be obtained as compared to with enzymatic dissociation. The advantages are that exposure to enzymes is avoided, the follicles retain theca and basement membrane, small and large follicles can be collected and larger and fibrous ovarian tissue can be dissected.

2.3.2 Follicle Cultures

2.3.2.1 Multiple Follicle Culture. Whole ovaries or ovarian fragments are cultured to resemble *in vivo* like conditions and is typically used to study the effects of ovulation and hormone responsiveness [20,29]. This type of culture is complex and results are difficult to analyze since ovarian fragments and whole ovaries contain viable follicles of varying sizes and stages of growth coexisting with apoptotic follicles and many extrafollicular cell types. Defining the local biochemical and hormone control pathways is not very feasible, and a loss in viability occurs with larger tissue fragments due to a lack of oxygen [18]. Nevertheless, whole ovarian or ovarian fragment culture has been successful with newborn mice [20].

Follicles, attached and not attached to each other, are also co-cultured. These follicles are either cultured for a short period (72 hours or less) to examine steroid production and hormone responses or for a longer period (6 to 12 days) to examine growth, endocrinology, and mostly interfollicular communication [18]. Non-attached follicles usually grow independently of each other but aggregate in culture, and growth rates of similar sized follicles tend to vary. On the other hand interactions between attached follicles in culture have been more noticeable, especially in mice [30].

2.3.2.2 Individual Follicle Culture. Follicles are commonly cultured individually in a well to investigate metabolism, hormonal influences, and oocyte development. Follicles that are not spherical, do not have a centrally located oocyte, or have a dark granulosa layer, which may be evidence of apoptosis, are considered irregular and are usually avoided during isolation from the ovary. Early preantral follicles, or follicles just after the primordial stage that have initiated their growth *in vivo*, are generally chosen for culture. In the mouse model, the largest size that a follicle generally grows to is approximately 500 μm in diameter, which is almost the size of a preovulatory follicle found in vivo [18].

Maintaining follicle integrity *in vitro* has been and still is a challenge. For instance, it is reported that approximately 20 % of follicles grow irregularly and damage during isolation from the ovary [22,31] and apoptosis during culture are usually to blame. In addition, follicles often loose their spherical structure by flattening and even rupturing during culture. For instance, when placed in a culture well, follicles initially settle to the surface and flatten. Sometimes a follicle will rupture at a defect in the basement membrane and as a result, the granulosa tissue will exit the follicle and grow over the basement membrane. Although flattening and rupture is often mentioned in the literature, there has been very little investigation on the issue.

A positive aspect of individual follicle culture is that between 40 % to 75 % of follicles cultured *in vitro* undergo antrum formation and continue growth, which exceeds the anticipated amount that occurs *in vivo* (20 %) in mice at an age of 28 days [18]. In addition, when placed in culture media, individual follicles respond to gonadotrophins, or certain hormones released from the pituitary gland, which stimulate their growth and development. For instance, when follicles

are cultured in media containing FSH, glucose utilization, steroid production, increases in granulosa cell density, and antrum formation are stimulated [18].

2.4 Tissue Culture in a Rotating-Wall Vessel (RWV)

2.4.1 Introduction

The disadvantages of conventional cell and tissue culture methods are primarily associated with sedimentation, damaging shear stress, turbulence, and inadequate oxygenation [32]. Gravity induces cells to sediment to the bottom of a culture well or vessel where attachment occurs and cell morphology becomes distorted. As a result, culturing cells in microgravity has recently been acknowledged as a method to maintain the three-dimensional structure of cells and tissue in culture by a lack of gravity induced sedimentation. In order to successfully culture cells or tissue in simulated microgravity, the cells or tissue, usually cultured with microcarriers, must be suspended in the culture medium without turbulence or damaging shear stresses while delivering oxygen and nutrients.

In 1990, the Biotechnology Group at NASA's Johnson Space Center in Houston, TX [33], developed the Rotating-Wall Vessel (RWV), a class of bioreactors designed for suspension cultures of animal cells in simulated microgravity [34]. Originally intended to protect fragile cell cultures from shear forces generated during space shuttle launches and landings, the RWV was

eventually tested for cell suspension cultures on ground [35]. It was demonstrated that cells aggregate and form large tissue-like structures in the ground based RWV cultures [35]. As a result, proliferation, differentiation, and cell-cell interactions of multiple cell types cultured in the RWV have been investigated.

Currently, the RWV has a broad range of applications, which include cancer research, in vitro toxicology research, and tissue engineering. For instance, rat bone marrow stromal cells were co-cultured in a RWV with microcarrier beads [36]. Complex structures connected by a collagen-rich extracellular matrix and containing calcium phosphate deposits distributed in the newly developed matrix between the beads, were observed [36]. In another study, rat adrenal chromaffin cells were co-cultured in a RWV with a microvascular endothelial cell line and after 20 days in culture self-forming "organoids" were observed [35]. These structures contained adrenal cells surrounded by an extracellular matrix consisting of fibronectin and type IV collagen, and grew to sizes similar to a normal rat adrenal gland [35].

2.4.2 Design Features of the RWV

NASA developed the RWV while trying to optimize the mechanical conditions found in other suspension culture devices, such as a mixing flask or an air lift bioreactor, which have large shear effects and turbulence due to their methods of agitation. The RWV, shown in Figure 3, is a cylindrical culture vessel equipped with a silicone membrane oxygenator positioned either coaxial as a core or flat on one side. This device was built to suspend cell and tissue constructs in a completely fluid filled culture vessel by rotating it around its horizontal axis at a rate that

prevents sedimentation and maintains minimal shear stresses. As the vessel wall rotates, the culture medium inside accelerates until everything within the vessel is rotating as the same angular rate as the wall [37].

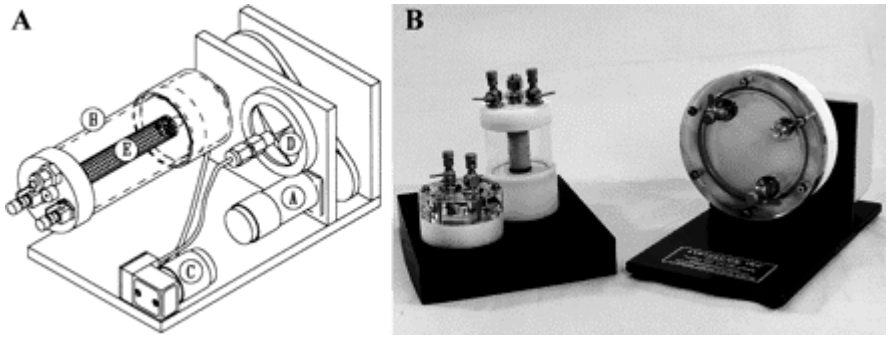

Figure 3 Rotating-wall Vessels

(33)

Left (A): This is a schematic of the rotating-wall vessel with a coaxial oxygenator membrane in which (A) a motor drives a belt to rotate (B) the culture vessel, and (C) an air pump draws incubator air through a (D) 0.22 μm filter and discharges it through a rotating coupling on the shaft that holds the vessel. The (E) oxygenator surrounds the center core.

Right (B): This photograph is of the RWV with an oxygenator positioned *(left)* coaxial and *(right)* on the side.

Culture medium is mixed by the vessel rotation and therefore eliminates the necessity for turbulence inducing impellers or stirring vanes used in other bioreactors to mix medium. In addition, the RWV is completely filled with culture medium to eliminate a headspace. In vessels that are incompletely filled with medium, such as roller bottles, the gas in the headspace creates turbulence and an increased formation of bubbles in the medium, which are a source of shear [33]. Bubbling and sparging of air is commonly known as a source of mechanical damage to cells in culture. However, in the RWV, large volumes of oxygen are delivered continuously along the oxygenator membrane in which dissolved gases are utilized [33].

Since 1992 the design of the RWV is being further optimized by Synthecon, Inc., a spin off company formed by the inventors of the bioreactor [38]. For instance, a perfusion system was incorporated into the design to continuously flow culture medium through the vessel and the oxygenator [39]. It is also equipped with inline pH, oxygen, and glucose monitors for feasible biochemical analysis.

2.4.3 Principles of Suspension Culture in the RWV

In the RWV, a construct consisting of cells or tissue, can be suspended and grow in three dimensions by balancing the gravity-induced sedimentation, the centrifugal effect caused by the rotation of the vessel wall, and the hydrodynamic drag due to the fluid circulation, as shown in Figure 4 [32,40]. Drag force is exerted on a construct when there is a relative motion between the construct and the rotating culture medium and can cause shearing [32]. One study has shown that if the density of a construct is less than the density of the surrounding fluid, the construct will

eventually migrate to an equilibrium state in the fluid [32]. In other words, the construct will have a circular orbit about the horizontal axis. However, if the density of a construct is greater than the density of the surrounding fluid, the construct will eventually migrate away and collide with the wall of the vessel [32]. If the density of a construct is similar to the density of the surrounding fluid, the construct will generally move with the bulk fluid [32]. This is, however, an unstable situation because with small disruptions, the construct will migrate to different locations within the vessel.

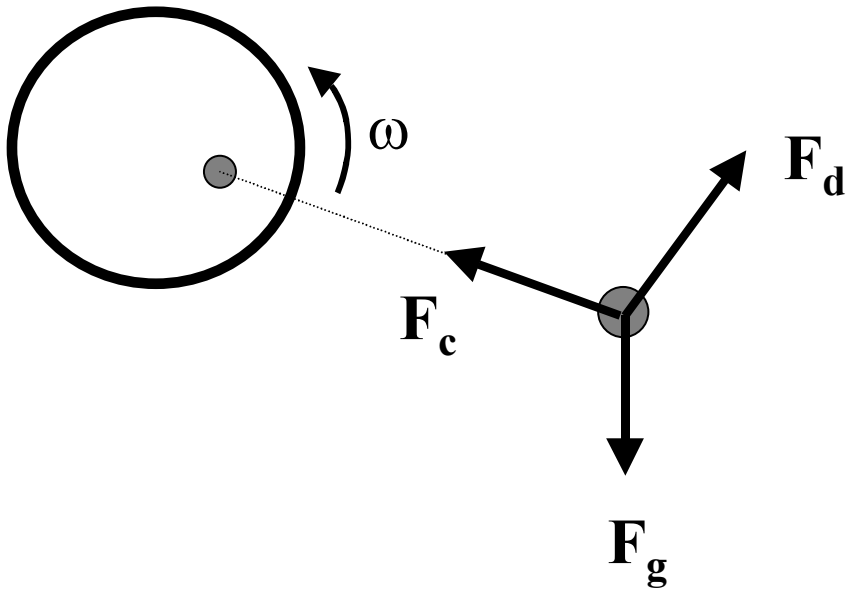

Figure 4 Forces on a Suspended Construct During RWV Culture

(32,41)

F_g is the force due to gravity induced sedimentation. F_c is the force due to the centrifugal effect caused by vessel rotation, ω. F_d is the force of drag due to the fluid circulation, also called hydrodynamic drag force.

2.5 Cell and Tissue Microencapsulation

2.5.1 Introduction

Cells typically exist in an immobilized state *in vivo* and quite often depend on their organization and interactions with adjacent cells. Biotechnologists utilize immobilization techniques to protect cells and tissues from foreign conditions, washout, shear stress, and immunological rejection and also to provide an organization and structure that enables interactions between neighboring cells [42]. The most common objective of cell immobilization is to offer a controlled and effective treatment of severe disorders in humans that are mostly associated with subnormal or deficient metabolism and cell secretions [43]. These diseases include multiple sclerosis, diabetes, blood disorders, Parkinson's disease, hemophilia, Alzheimer's disease, and hepatic failure.

Microencapsulation is a technique in which cells, tissues, or any biologically active component is immobilized in hydrogel beads or microcapsules. Cells are suspended in a hydrogel or a membrane forming material and then formed into microcapsules. The most common technique to produce microcapsules is to form porous gel beads from liquid droplets. These beads are sometimes coated with a polymer to further protect the cells and help prevent the beads from breaking down. Applying an appropriate capsule membrane biomaterial is essential in achieving success with cell encapsulation. This biomaterial must be non-toxic and biocompatible, mechanically and chemically stable, and permeable depending on the environment and cell type.

2.5.2 Microcapsule Diameter

The diameter is one of the most important properties of a microcapsule. The thickness of the gel affects mass transfer, but also provides support to the cells or tissue. It is important to define an optimal size according to the application and to use a droplet formation method that provides the proper diameter.

A microcapsule or bead must be large enough to contain the cells or tissue and allow for growth. However, it is necessary that implantable microcapsules be smaller than one half of the internal diameter of the injection needle [43]. Although larger beads are easier to handle, there may be a significant internal dead volume leading to mass transfer limitations. In addition, when culturing in a bioreactor, shear and abrasion effects increase with increasing microcapsule diameter [44].

Obtaining the optimal diameter size is usually a compromise. In fermentation a diameter of approximately 2 mm is typically suggested to facilitate handling, while a diameter of 800 μm is necessary to reduce mass transfer limitations [42]. In transplantation, microcapsule diameters are typically within the range of 300 to 800 μm, and it is often suggested to limit the size to less than 500 μm [42].

2.5.3 Droplet Formation Techniques

2.5.3.1 Introduction. Capsules or gel beads are generally formed by a droplet extrusion technique, as shown in Figure 5. These involve forcing a liquid, containing a suspension of cells or tissues, through a nozzle or needle and into a beaker containing a solution that will solidify the droplets into gel beads or capsules. As the flow rate of the liquid is increased, a droplet will stretch prior to detachment until the liquid forms a jet or a continuous stream from the tip of the needle or nozzle and naturally will break into small droplets [(42)]. In choosing the proper droplet formation method, certain parameters must be taken into account, such as: desired mean diameter, acceptable size dispersion, production scale, and the maximum shear stress that the cells or tissue can withstand [(42)]. The following briefly describes common techniques currently applied to form droplets.

2.5.3.2 Droplet Formation By Gravity. The simplest way to form droplets is to allow a liquid droplet to fall from the tip of a needle or nozzle by only the forces of gravity. This technique generally provides droplet diameters of larger than 2 mm even when using the smallest needle diameters. As a result, droplet formation by gravity has received limited interest.

2.5.3.3 Droplet Formation Using Coaxial Air or Liquid Flow. A coaxial air stream or liquid jet stream around the needle is sometimes used to increase the force acting on the developing droplets. Many research laboratories utilize this technique since it provides gel beads or microcapsules with diameters as small as a few micrometers to one millimeter. For a given liquid concentration, type, and viscosity, the bead diameter depends only on the airflow for a given syringe diameter. For instance, when using alginate as the dropping liquid, bead diameters in the range of 0.2 to 3 mm can be produced when using a syringe diameter of 0.27 mm and an airflow up to 3 l/min [45]. Although very small bead diameters can be produced, the size dispersion increases significantly as the bead diameter is decreased [42,46]. Therefore, this technique is not typically considered for scale up, but is generally appropriate for laboratory experiments.

2.5.3.4 Droplet Formation Using Electrostatic Potential. Droplet diameter can also be reduced down to 200 μm if the drag force is replaced with a high electrostatic potential between the nozzle and the collecting solution [42,47]. The electrostatic potential can be applied between the nozzle and a stainless steel ring placed below the nozzle. As the electrostatic potential increases, the droplet size decreases. In this method, the liquid leaves the tip of the nozzle as a jet stream that breaks up into small drops. The main reason why small droplets form is that charged molecules migrate to the surface of the droplet and create repulsion between molecules at the air-liquid interface that is strong enough to counteract the surface tension force that holds the droplet at the tip of the nozzle [42]. Typical encapsulation methods apply an interaction between a high molecular weight polymer and a counter ion. In order to achieve small droplets,

it is important that the polymer and the counter ion have the same charge. Overall, this method generally provides a smaller size distribution than that obtained with coaxial airflow (standard deviation of approximately 15 %) [42] and is also mainly used in laboratory scale.

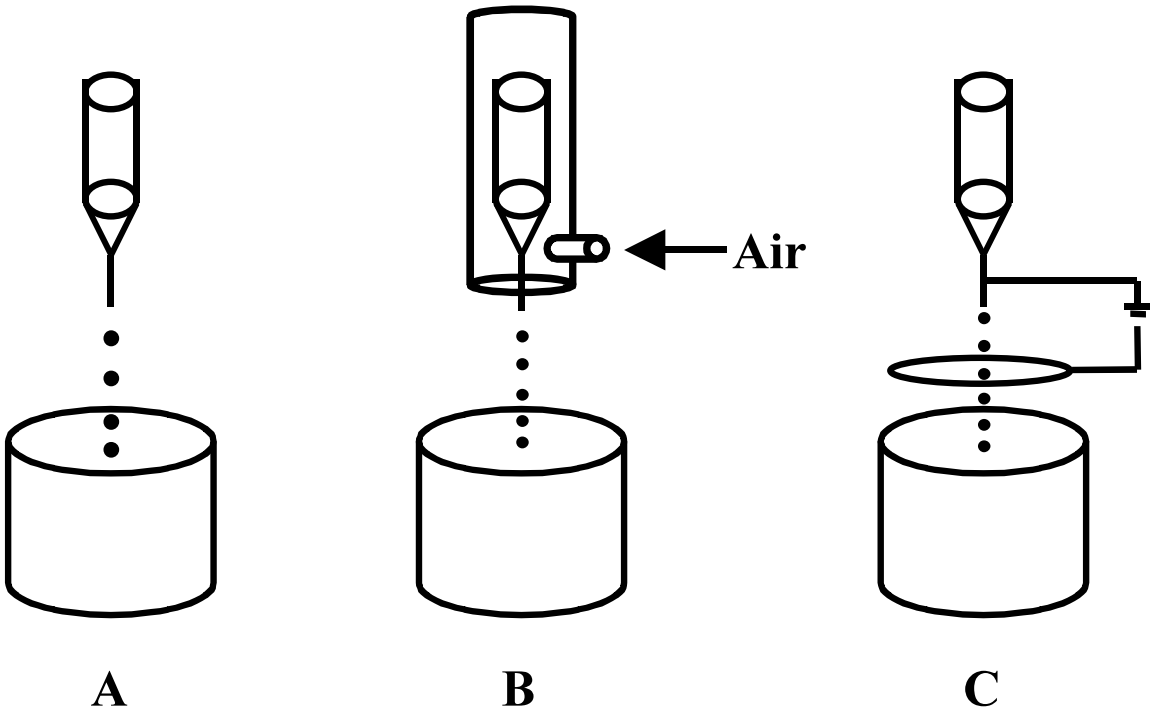

Figure 5 Droplet Formation Techniques

(42)

(A) By Gravity, (B) By Coaxial Airflow, and (C) Under Electrostatic Potential.

2.5.4 Cell Entrapment By Ionotropic Gelation

2.5.4.1 Introduction. Due to its simplicity and mild process conditions, entrapment of cells in hydrogel beads is the most commonly applied cell immobilization technique. Ionotropic gelation is a method of hydrogel bead entrapment that involves dropping a charged polymer into an opposite charged multivalent counter-ion solution [42]. This is an attractive method since it is performed under mild conditions that do not involve changes in pH or temperature, does not require toxic reagents, and is a simple and economical process.

2.5.4.2 Alginate. Alginate is the most widely used polymer for cell encapsulation due to its biocompatibility, high porosity, and ability to form gels under mild conditions [48,49]. It is a family of unbranched polysaccharides that are generally extracted from brown algae. Alginate is composed of 1,4-linked β-D-mannuronic (M) and α-L-guluronic (G) acid residues that exist in varying proportions and sequences [45,48,49]. For instance, the residues are arranged in the polymer chain as blocks, such as poly(mannuronic acid) or M blocks, poly(guluronic acid) or G blocks(M-M or G-G blocks) and poly(mannuronic-alt-guluronic acid) or MG blocks, as shown in Figure 6 [45,50].

The composition and sequence of the polymer chain affects the properties of an alginate gel. Differences in flexibility arise from restrictions surrounding the carbon-oxygen bonds that join the monomers [50]. The linkage of G residues, which are buckled, induces more steric hindrance than the linkage of M residues, which are flat [50]. Therefore, alginate polymers with a

greater M content are more flexible than alginate polymers with a greater G content [50]. On the other hand, alginates with a greater G content and longer G blocks offer higher mechanical strength, lower gel shrinkage, more stability, and higher porosity [45,50]. Alginate forms a gel by a highly cooperative reaction in which divalent cations, such as Ca^{2+}, Sr^{2+}, and Ba^{2+}, tightly bind within G blocks from two different chains [50]. The diaxially linked G residues form electronegative cavities that offer binding sites for ions, giving rise to junction zones within the gel, and also provides a more open pore structure [50]. As a result, the length of the G blocks has a significant impact upon gel formation.

The encapsulation process involves simple steps under mild conditions that are compatible with most living cells. Cells are mixed with a sodium alginate solution, taken into a syringe with a needle, and then the mixture is dripped into a solution containing divalent or trivalent cations, typically a calcium chloride solution. The cations, such as calcium ions, instantly diffuse into the alginate droplets to form gel beads that immobilize the cells into a lattice of ionically cross-linked alginate [45].

A disadvantage of calcium alginate gels is that substances that have a high enough affinity for Ca^{2+}, such as phosphate, citrate, lactate, or EDTA, will lower the stability of the gel by sequestering the cross-linking calcium ions [45]. The gel can also be destabilized by the presence of high concentrations of non-gel forming cations, such as Na^{+} and Mg^{2+}; however, this problem can be overcome by use of a stronger gelling agent, such as barium or aluminum ions, and a higher G content alginate [45].

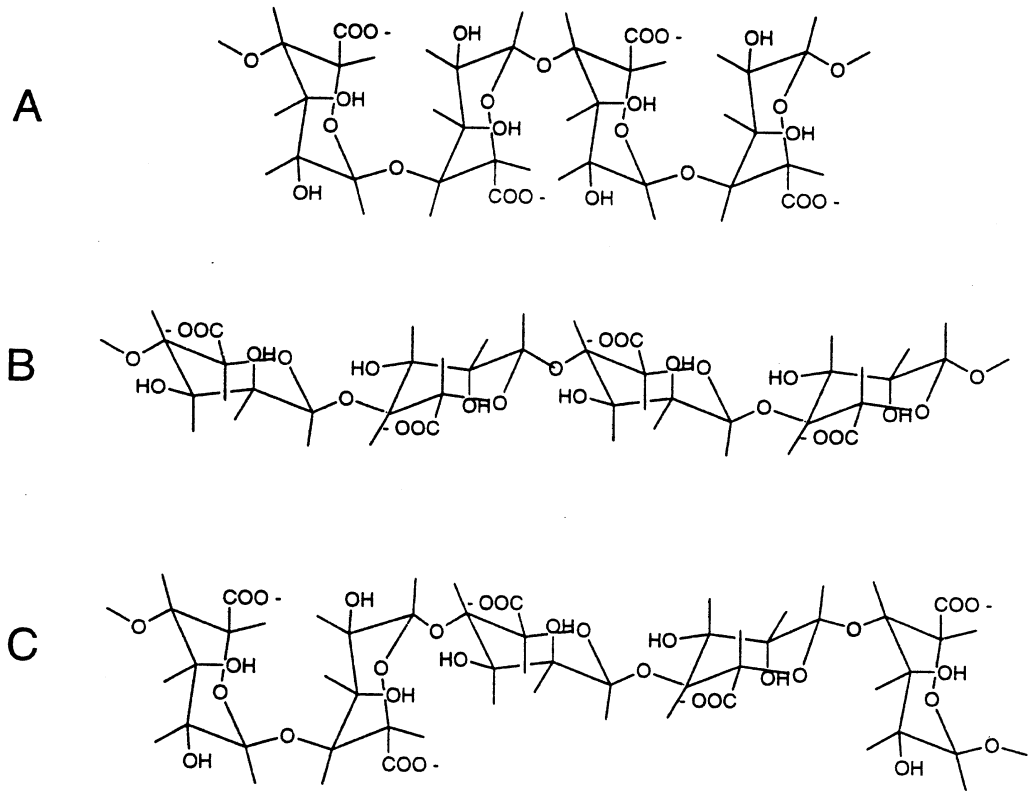

Figure 6 Alginate Chemical Structures

(50)

(A) poly(guluronic acid) sequence, (B) poly(mannuronic acid) sequence, and (C) poly(mannuronic-alt-guluronic acid) sequence.

2.5.4.3 Other Biomaterials. Other biomaterials applied in ionotropic gelation include chitosan and pectins. Chitosan is a polyglucosamine polysaccharide from chitin [51]. The gel formation process involves dropping a chitosan solution into a solution containing polycations, such as a phosphate solution [42]. Unlike alginate, chitosan is stable in phosphate buffer. Although alginate and chitosan beads have similar mechanical stability, the use of chitosan for cell encapsulation is limited due to being water soluble only for pH levels lower than 6.5 and causing a loss of cell viability by interactions with cell membranes [42].

Pectins are acidic polysaccharides, obtained from plant cell walls that form a strong gel when dropped in solutions containing calcium or aluminum ions [42]. The sensitivity of calcium pectate gel beads to calcium chelators or other competitors is much less than with calcium alginate gel beads. In addition, beads have been produced from pectate-alginate mixtures and were reported to be highly stable [42].

3.0 RESEARCH OBJECTIVES

3.1 Conventional Follicle Culture

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, or preantral follicles. Preantral follicles grow at a slow rate and their small size makes it difficult to isolate them for culture [11]. 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 [16,17,18,19], shown in Figure 7, often occurs around 48 to 72 hours of static culture in a tissue culture well and is rarely addressed for investigation. Follicle rupture is characterized by the disruption of the follicle and the movement of the 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.

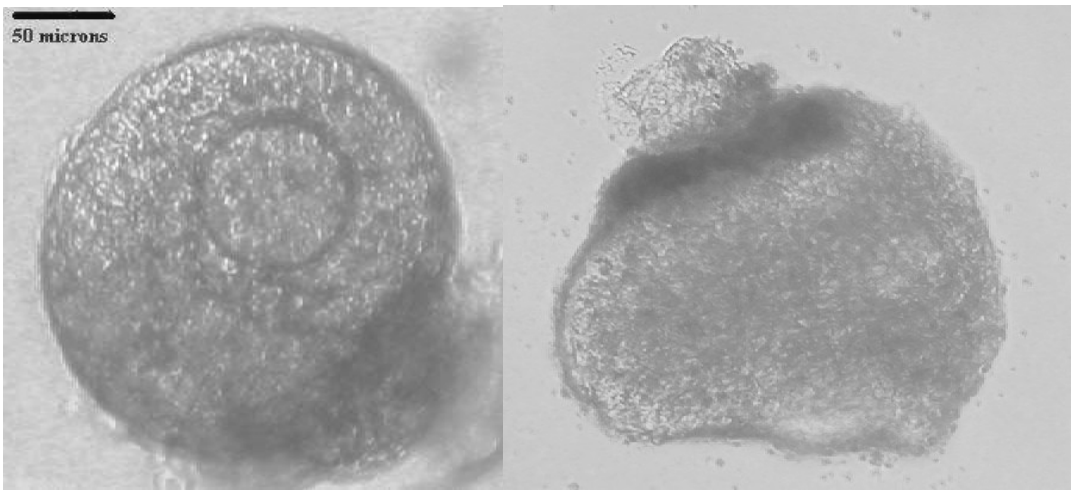

Figure 7 Flattened and Ruptured Rat Preantral Follicles

Left: Flattened; *Right*: Ruptured.

Follicle flattening and rupture could be the result of a non-uniform environment of a follicle resting in a well, a nutrient transfer limitation, 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 [8,18,52], 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.

3.2 Suspension Culture

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. Although both devices are designed to prevent cells and

tissues from settling, the drag force of the circulating media in the rotating-wall vessel exerts potentially damaging shear stress [32].

Ovarian follicles are extremely fragile and, mechanical damage may be experienced due to shearing. It has been reported that damage and a loss of viability occur when anchorage dependent mammalian cells are exposed to shear levels in the range 3 to10 dyne/cm^2 [53,54]. It also has been reported that proliferation, morphology, and function of BHK-21 kidney cells are negatively affected even at a much lower shear stress level of 0.92 dyne/cm^2 [55]. In order to enhance growth and differentiation in three dimensions, shear levels of 10^{-2} dyne/cm^2 are recommended when culturing any anchorage dependent mammalian cells [53].

3.3 Microencapsulation

Semipermeable hydrogels are used to encapsulate tissue, functioning as a barrier from infection and mechanical stress [56,57]. 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 [45]. 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 [56,58]. 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 [45]. 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 [45]. 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 in water [59], while there is some resistance for large proteins of molecular weight greater than 3×10^5 [45]. 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.

4.0 EXPERIMENTAL PROCEDURES

4.1 Follicle 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 [9,60,61]. 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.

As illustrated in Figure 8**,** 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 as shown in Figure 8. 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.

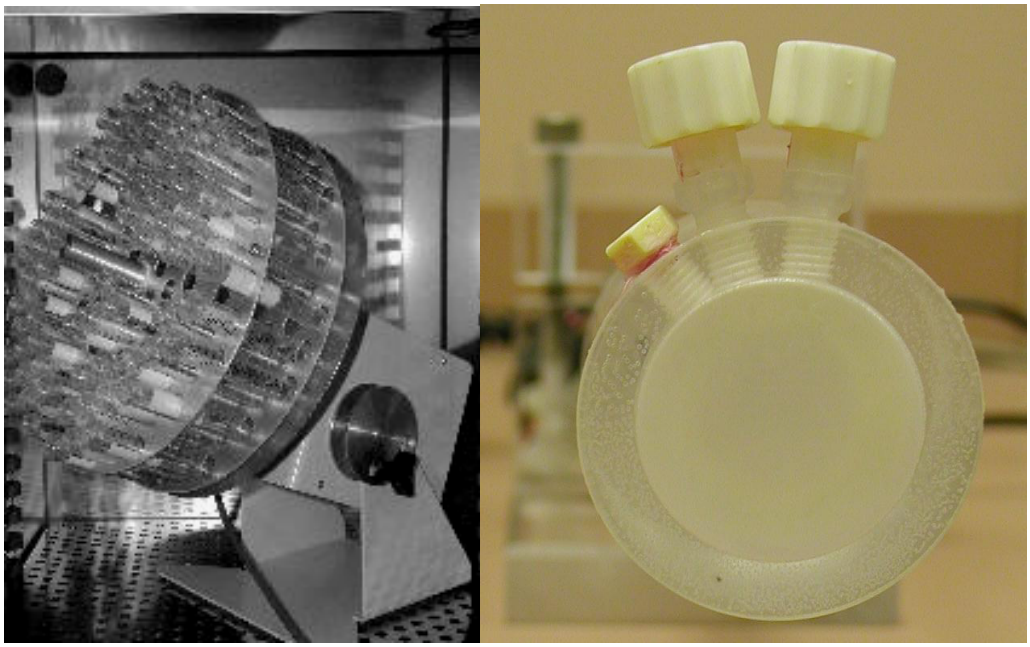

Figure 8 Suspension Culture Systems

Left: Orbiting Tubes; *Right:* Rotating-wall Vessel.

4.2 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.

4.3 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. As illustrated in Figure 9, 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 Figure 9.

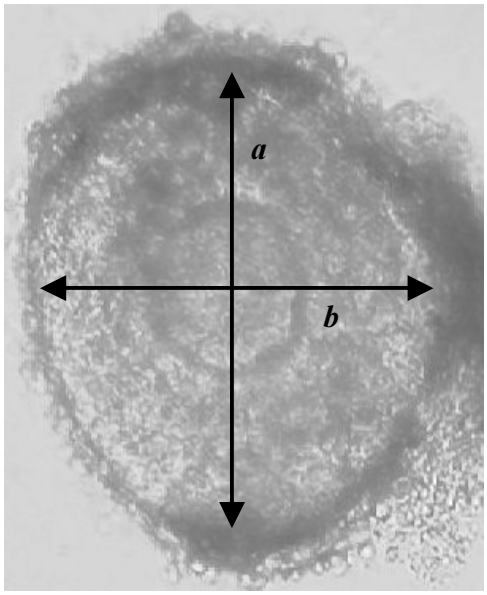

Figure 9 Degree of Flattening

Follicle diameter is measured as the distance between the basement membrane from two sides. When flattening occurred, two diameters, *a* and *b*, were measured, and the degree of flattening was calculated as shown in Equation 1.

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

4.4 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 [62]. 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.

4.5 Follicle Density and Maximum Shear Stress Calculation

For a follicle to move in a circular orbit during culture in a rotating-wall vessel, the density of the follicle, ρ_f, must be less than the density of the culture medium, ρ_m, otherwise, if it is larger the follicle may eventually collide with the wall [32]. Stokes law for sedimentation, Equation 2, was rearranged to solve for an average density of a rat preantral follicle, ρ_f. An average settling velocity, v_s, was calculated from ten follicles by measuring the time taken for each one to fall through a measured distance in the bottom half of a cylinder filled with water at 37 ^{0}C [41]. All other parameters, such as the acceleration due to gravity (980 cm s $^{-1}$), g, follicle

diameter (0.0148 cm), d, culture medium density at 37 ^{0}C (used value for water, 0.99 g cm $^{-3}$), ρ_m, and culture medium viscosity at 37 ^{0}C (used value for water, 0.007 g cm $^{-1}$ s $^{-1}$), μ, are known. The calculated follicle density was then compared to the culture medium density to determine whether a preantral follicle is in relative motion with the medium during culture in the rotating-wall vessel, and if so, it may experience some shear damage.

It is recommended to maintain shear stress levels as low as 10^{-2} dyne/cm^2 to allow for three-dimensional growth and differentiation when culturing anchorage dependent mammalian tissue [53,54]. If shear stresses in the range of 3 to10 dyne/cm^2 are exerted on mammalian tissue, mechanical damage and a loss of viability will occur [53]. Even when shear stresses as low as 0.92 dyne/cm^2 are applied, proliferation, morphology, and function are halted in culturing of BHK-21 cells of the kidney [55]. Therefore, we calculated an estimate for the maximum shear stress exerted on a follicle while it settles through media to determine if that value meets the recommended level of 10^{-2} dyne/cm^2.

The maximum shear stress, τ_{max}, exerted on the follicle, which is a function of the viscosity of the culture media, μ, settling velocity of the follicle, v_s, and diameter of the follicle, d, can be calculated based on Stokes hydrodynamic drag force, F_d, (Equation 3) acting on the surface area of a spherical particle, S, (Equation 4) as long as the Reynolds number, Re, is less than 1 or the inertial forces are negligible [33,41,63,64]. An average maximum shear stress was then calculated from the ten preantral follicles, as shown in Equation 5.

Equation 2 $$v_s = 0.056\, g\, d^2\, (\rho_f - \rho_m) / \mu$$

Equation 3 $F_d = 3\ \pi\ \mu\ d\ v_s,$ *if Re < 0.3 for spherical particles*

Equation 4 $S = \pi\ d^2,$ *for a spherical particle*

Equation 5 $\tau_{max} = F_d / S = [\ (3\ \mu\ v_s)\ /\ d\ ]$

4.6 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.

4.7 Statistical Analysis of Data

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.

5.0 RESULTS AND DISSCUSION

5.1 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 10). Follicles cultured in the presence of FSH (CCS+FSH) had a 20.5 % ($p<0.05$) increase in average diameter during the culture period.

5.2 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 11 and 12). 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.

5.3 Follicular Flattening and Rupture in Suspension Culture Systems

5.3.1 Orbiting Test Tube - Suspension Culture Systems (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 10, 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 1). 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.

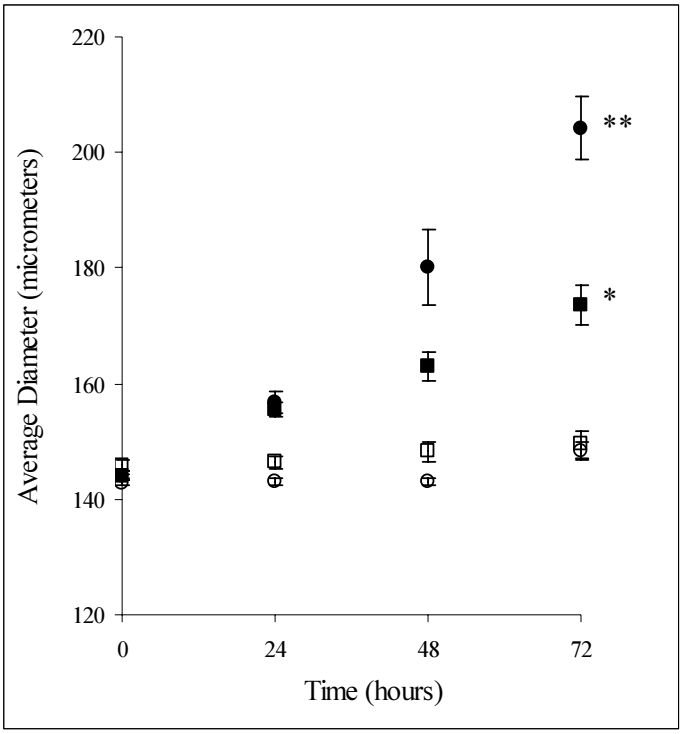

Figure 10 Growth of Rat Preantral Follicles

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.

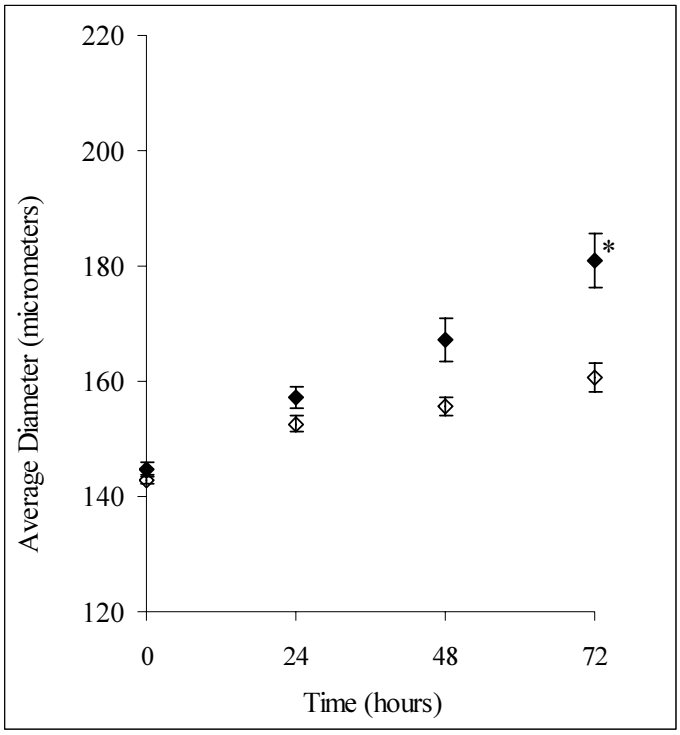

Figure 11 Growth of Follicles Cultured in the Conventional Culture System

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. One asterisk, ∗, represents a significant difference ($p < 0.05$) from the CCS+FSH spherical group.

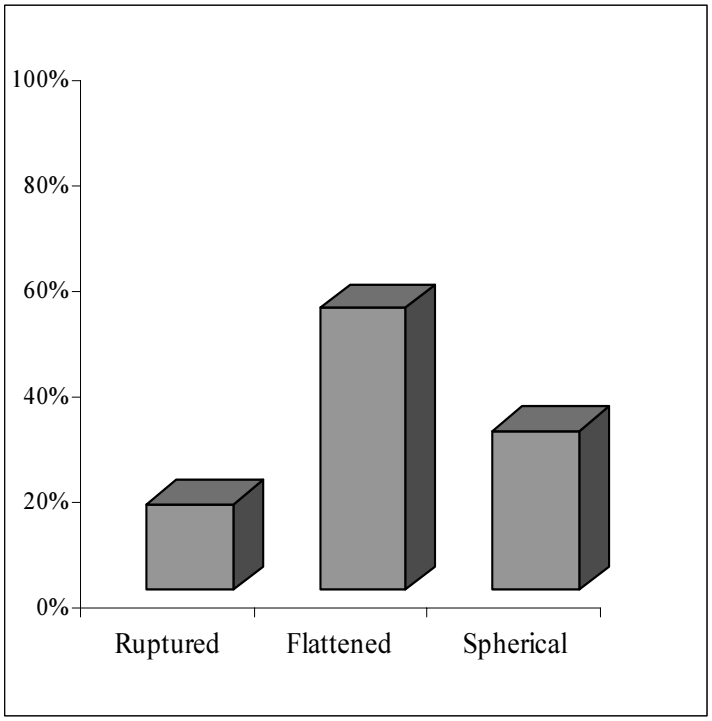

Figure 12 Morphology of Follicles Cultured in the Conventional System with FSH

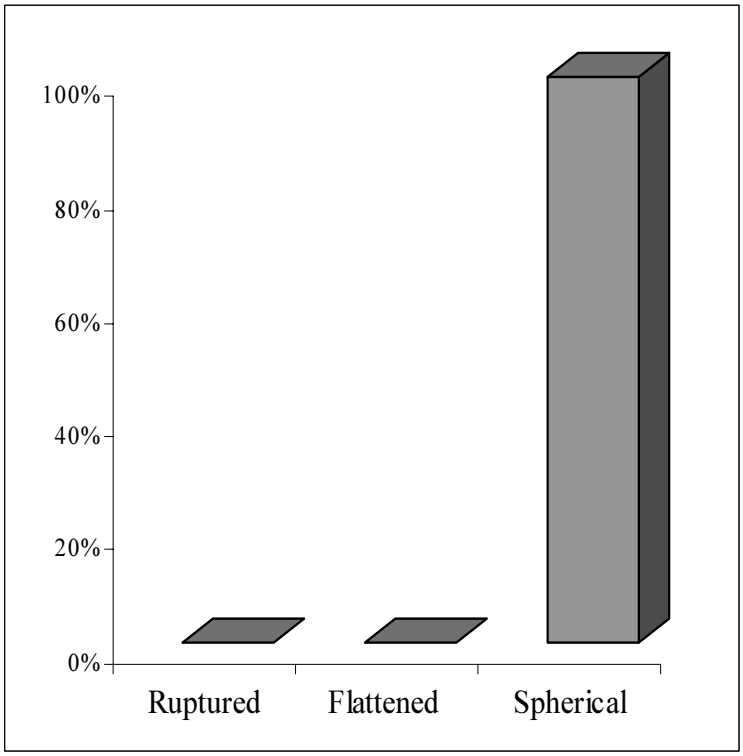

Figure 13 Morphology of Follicles Cultured in Orbiting Test Tubes with FSH

Table 1 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*

5.3.2 Rotating-wall Vessel - Suspension Culture Systems (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. We decided therefore to compare the difference in density between a preantral follicle and the culture medium and also determine the maximum shear stress exerted on a rat preantral follicle while settling through culture media. By measuring an average settling velocity of 0.128 cm/s for preantral follicles, with an average diameter of approximately 148 μm, the density of a follicle, ρ_f, was calculated as 1.07 g cm^{-3}, which is greater (0.08 g cm^{-3}) than the

density of the culture medium at 37 ^{0}C, ρ_m. The maximum shear stress, τ_{max}, exerted on a follicle was calculated as 0.18 dyne/cm^2. This value is 18 times greater than what is recommended for maintaining growth and differentiation of anchorage dependent mammalian cells [53].

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 74.5 % fragmented and 16.4 % did not grow, but interestingly 9.1 % of the follicles experienced a rapid diameter increase of 29.3 % (Figure 14). These follicles had spherical morphology (Figure 15), but the darkened appearance of the granulosa layer suggests that the follicles may have experienced some damage, and could be a result of shearing (Figure 16). Due to the difference in density between the follicle and culture medium, follicles may have migrated to various positions and even collided with the vessel wall.

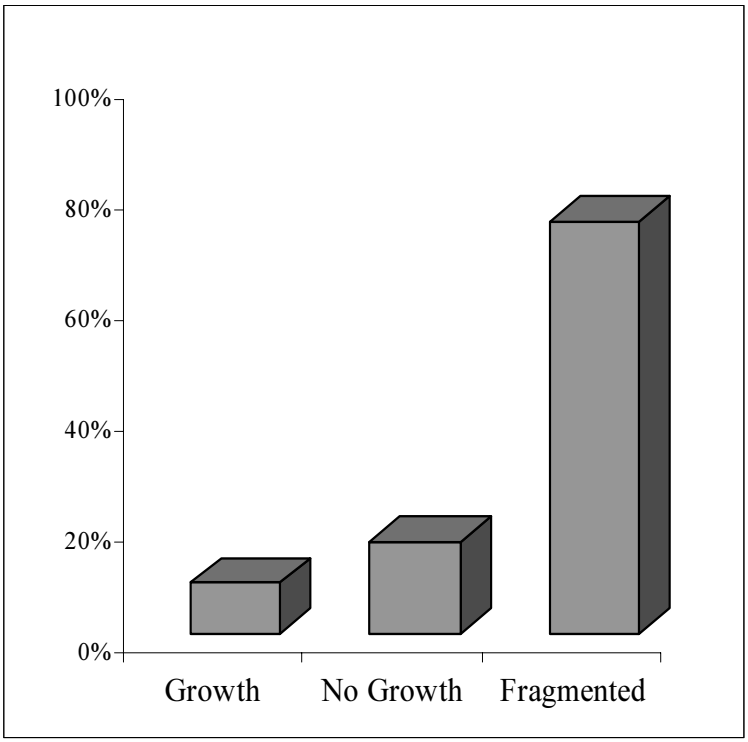

Figure 14 Growth of Rat Preantral Follicles Cultured in Rotating-wall Vessels

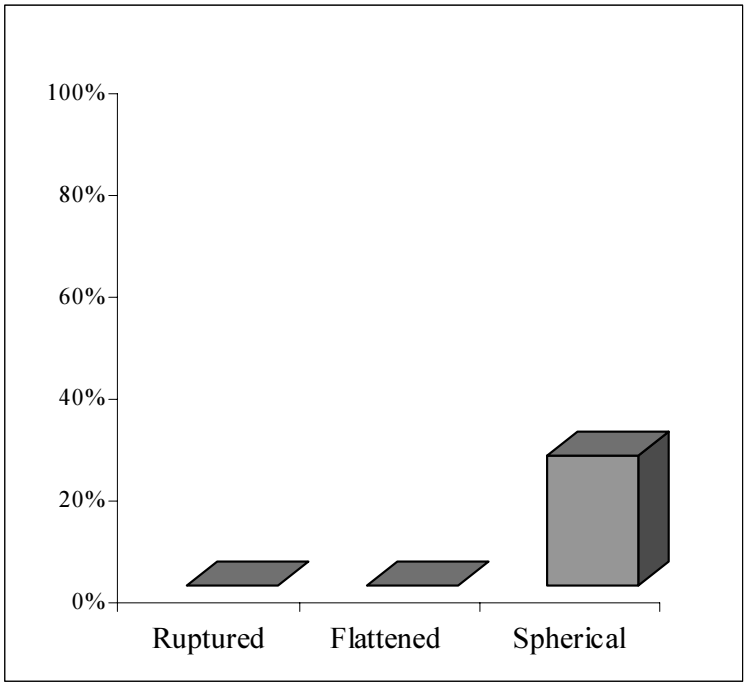

Figure 15 Morphology of Follicles Cultured in the Rotating-wall Vessels with FSH

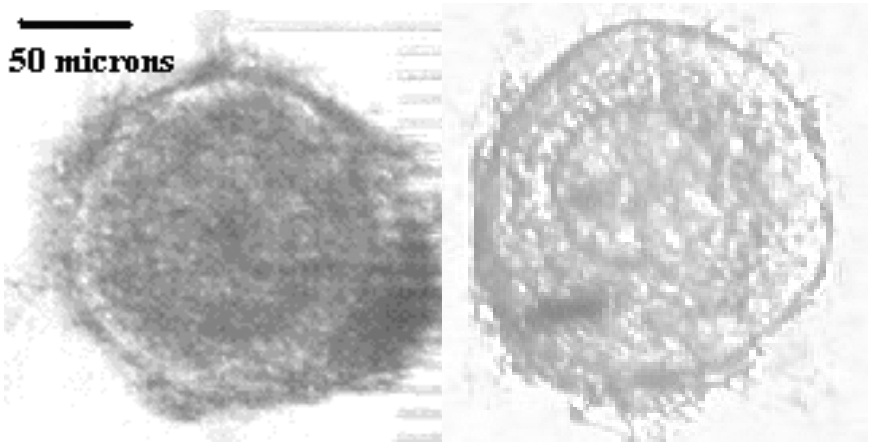

Figure 16 Rat Preantral Follicles Cultured in Rotating-wall Vessels

Left: Follicle with darkened granulosa cells.

Right: Follicle with no apparent damage.

5.4 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. 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 [42,45].

Our data show an average increase in diameter of 24.1 % for rat preantral follicles (Figure 17) and 29.3 % for mouse preantral follicles (Figure 18), 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 (Figure 19). They were more spherical and appeared similar to natural morphology, as shown in Figure 20. 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.

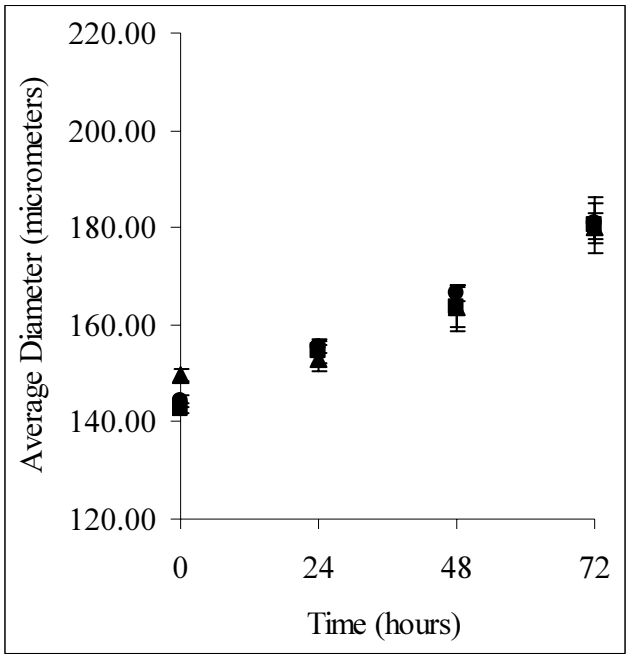

Figure 17 Growth of Encapsulated Rat Preantral Follicles

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.

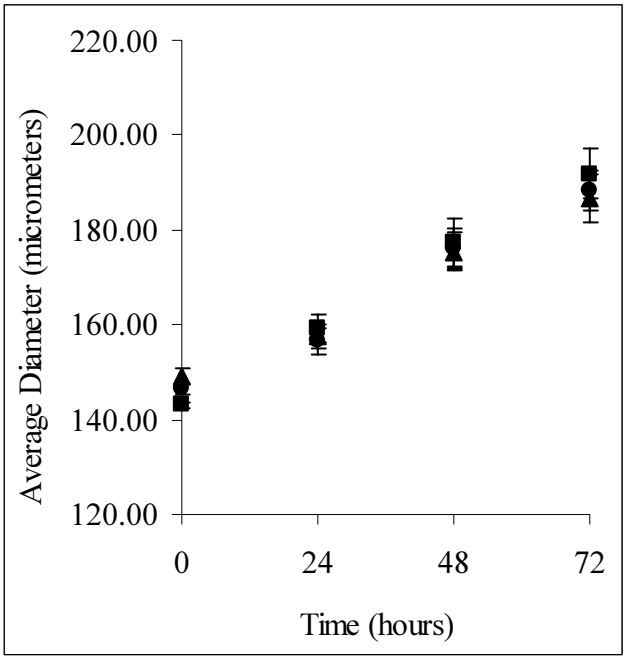

Figure 18 Growth of Encapsulated Mouse Preantral Follicles

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.

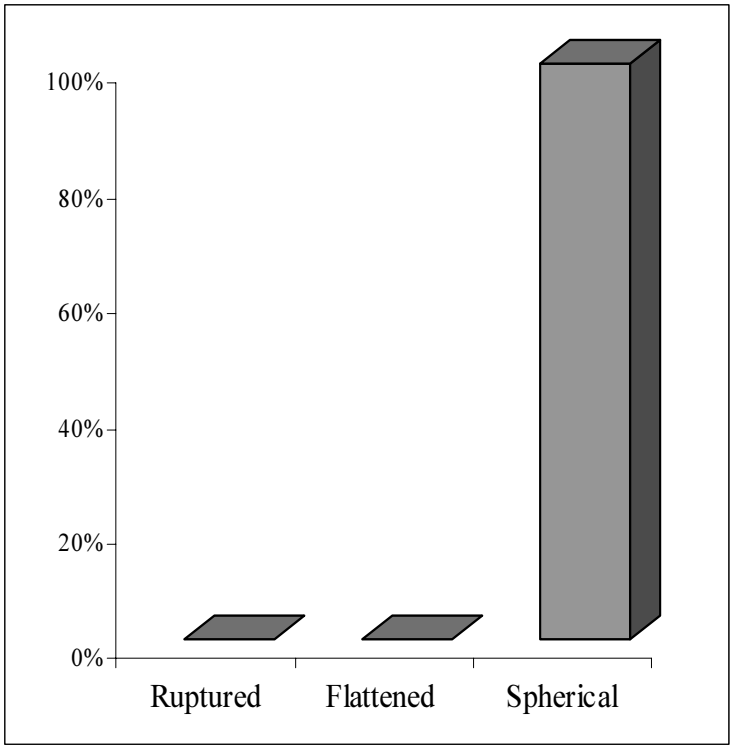

Figure 19 Morphology of all Encapsulated Follicles Cultured with FSH

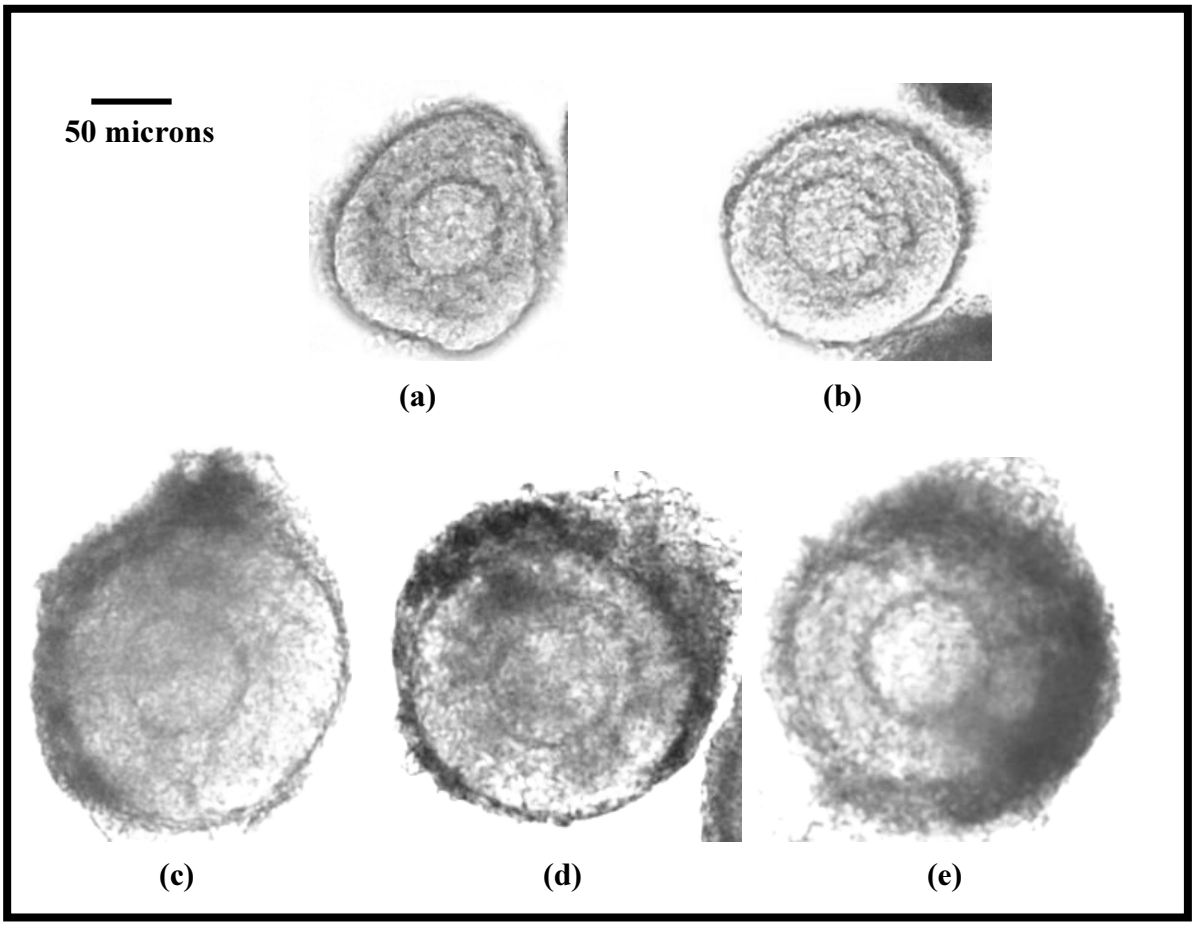

Figure 20 Rat Preantral Follicles

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.

6.0 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 [22,65], similar success with species that have larger follicles has not been reported. The rate of follicle development and ultimate size varies between species [10] 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.

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