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NOVEL IN VITRO MODEL TO GROW AND CULTURE OVARIES

MID-SEMESTER REPORT
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ABSTRACT

As cancer chemotherapy treatments become more sophisticated, there has been an increase in cancer survivors. With more patients surviving, long-term side effects of chemotherapy have become prevalent. One of these side effects is primary ovarian insufficiency (POI), which is seen in 40% of reproductive age breast cancer survivors. Studies have shown that doxorubicin chemotherapy is associated with ovarian insult. The exact model of this insult has not been determined. To investigate this phenomenon further, the design of a novel device for studying ovaries *in vitro* was proposed by the client, Dr. Sana Salih. Dr. Salih is investigating the effects of DXR on ovarian tissue and seeks a device to study the mechanisms of this ovarian insult. Motivation and design specifications were presented. Several design alternatives for culturing cow ovaries were considered and a final design was proposed. It was determined that a bioreactor providing the ovary with a carefully controlled environment would fulfill the design requirements. The bioreactor proposed provides nutrients to the ovary using an arterial cannula. Future work includes fabrication, assembly, and viability assessment. Ultimately, this project will be integrated with another project proposed by Dr. Salih focusing on DXR delivery into the ovary.

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BACKGROUND

As the treatment for various types of cancer continues to improve, survival rates have steadily increased over the past 20 years [1]. This has been due to advances in therapies such as radiation therapy and chemotherapy. These innovations in treatment allow more patients to live much longer, but unfortunately, also have been shown to lead to subsequent complications. For women undergoing chemotherapy, one such potential consequence is primary ovarian insufficiency (POI). The impact of this condition is felt throughout the medical community, as POI ultimately results in infertility in a large number of individuals affected. POI occurs in up to 40% of premenopausal breast cancer survivors and 8% of childhood cancer survivors. These demographics alone will constitute 1 in 800 women by 2020[2].

Primary Ovarian Insufficiency

In primary ovarian insufficiency, the ovary is unable to produce female hormones (such as estradiol) and alters the endocrine profile of the patient. The hormone imbalance can lead to menopausal symptoms and possible cessation of reproductive function [3]. POI is considered to be present in a woman if she is less than 40 years old, has amenorrhoea (or the absence of a menstrual period) for at least 4 months, and has follicle-stimulating hormone (FSH) levels in the menopausal range for consecutive months. Accordingly, primary ovarian insufficiency was previously referred to as premature menopause or premature ovarian failure. However, more indefinite patient outcomes required a more loosely defined term. Studies have shown that in 50% of cases, there was variable or unpredictable ovarian function. Additionally, 5 to 10% of sufferers were able to conceive and deliver a child despite receiving the diagnosis. These inconsistent outcomes validate the use of the term “primary ovarian insufficiency” to describe the continuum of ovary impairment [4].

Nonetheless, primary ovarian insufficiency is a serious unsolved problem and is the focus of many investigations. The disorder has a significant chance to cause complete sterility and has a pronounced effect on younger patients [5]. Further complications have been associated with POI, including osteoporosis and cardiovascular disease [2]. Patients also have an increased risk for neurological diseases, sexual disorders, and decreased libido. These factors provide motivation to determine the cellular cause of POI and develop measures to inhibit its development [5].

Ovarian Insult from Chemotherapy

Among other genetic and environmental associations with primary ovarian insufficiency, chemotherapy is a well-known cause [5]. Chemotherapy has been shown to cause ovarian insult, or progressive and irreversible damage to the ovarian tissue that leads to POI. Although the disorder itself has been well identified in chemotherapy patients, the mechanisms behind this toxicity are not understood. Therefore, efforts to protect the ovary from impairment have been largely unsuccessful [2].

In order to prevent cellular chemotherapy damage to ovarian function, an understanding of different ovarian cell types in relation to healthy folliculogenesis is required. As shown in **Figures 1** and **2**, folliculogenesis is the growth and development of follicles inside the ovary and is the principal pathway in ovulation. **Figure 1** illustrates the transition from primordial follicles (A), to primary follicles (B and C), to secondary follicles (D) and finally to antral follicles (E).

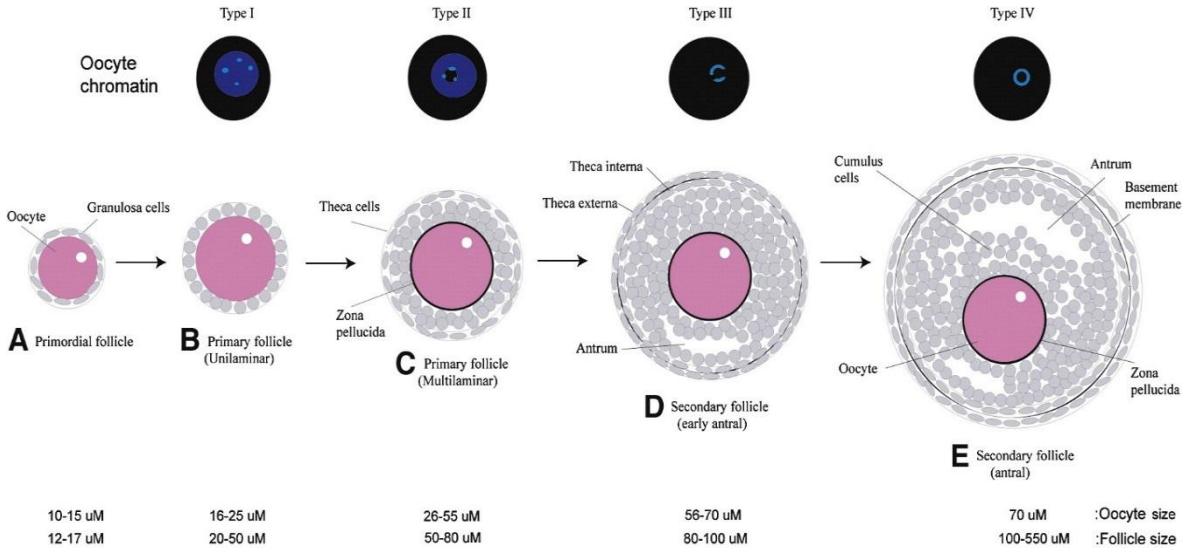

Figure 1. Follicular development [6].

Ovarian Physiology

The follicle is the fundamental and essential unit of female reproductive function. The main role of the follicle is to protect and develop the oocyte until it can be ovulated from the ovary as a precursor to a mature egg. Follicles start developing layers of granulosa cells (**Figure 1**) in the primordial follicle stage, and after receiving the necessary chemical or hormonal signals, grow specialized layers of theca cells. The granulosa cells provide nourishment to the oocyte, while the theca cells form extracellular matrix membranes around the follicle for protection and intercellular interaction [2].

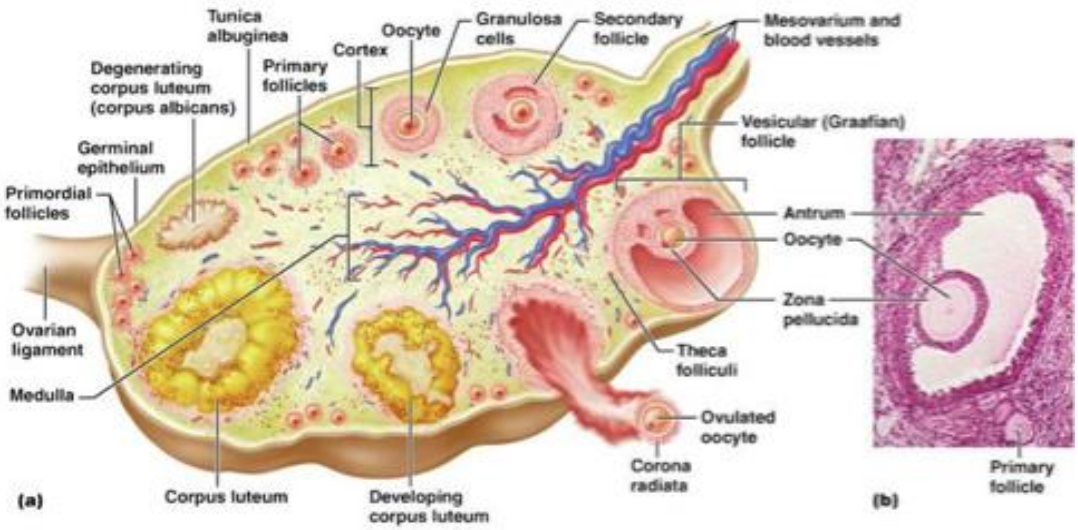

Figure 2. Ovary anatomy and follicle development [7].

Depicted in **Figure 2** is the spatial relation of folliculogenesis to the rest of the ovary. The medulla (center of the ovary) contains stroma cells and provides space for blood vessel formation. The follicles grow and develop almost exclusively around the outside edge of the ovary. Recognizing that the vasculature of the ovary does not directly reach the oocytes in follicles is especially important. The blood vessels only provide systemic circulation to the stroma and theca cells, while follicles depend on nutrient diffusion through the various cell layers to develop [2]. As the follicle grows into the antral phase (vesicular follicle), the theca cells fuse with the outer membranes of the ovary. The oocyte is then ovulated into the fallopian tubes and develops into a mature egg ready for fertilization [3].

Doxorubicin

Various investigations have shown that follicle and oocyte attrition is a direct cause of chemotherapy, and is linked to the diagnosis of POI. However, it remains unknown how these anti-cancer drugs interact with the cells in the ovary. Prominent hypotheses suggest that oocytes might be directly affected by the chemotherapy agents or are destroyed following the failure of the follicular cells around them [2]. Specifically of interest to researchers is the effect of doxorubicin (DXR). DXR is a chemotherapy drug used to treat about 50% of premenopausal cancer patients, and has been associated with a significant number of POI diagnoses every year [5]. In several cellular-scale investigations, DXR has had multiple effects *in-vitro* and is cell type- and DXR dose-dependent. Varying doses, in addition to isolated cell types, has been shown to result in cell apoptosis, autophagy, or necrosis. The effects of DXR have been documented in mice, including follicular attrition in as little as 12 hours post-injection and permanent reduction in ovary size up to 50%. At this point, the mechanisms behind these consequences and general oocyte or follicle damage due to DXR are not fully understood [2]. With this information, there is an opportunity to discover protective compounds or dosage techniques that could neutralize ovarian damage from DXR.

PROBLEM STATEMENT

All too frequently, female patients lose their reproductive capabilities as a consequence of undergoing cancer chemotherapy. Research has shown that doxorubicin chemotherapy causes ovarian insult, ultimately leading to primary ovarian insufficiency or complete ovarian failure. Currently, no systems exist to grow adult ovaries *in vitro* to test chemotherapy toxicity. This situation greatly limits ovarian research. Our client has proposed a system to test the effects of DXR on the ovary. This project will establish a novel in-vitro ovary culture system to maintain cell/tissue viability in order to facilitate assessment of chemotherapy toxicity. This device will allow for future mechanistic determination of DXR's effects on the ovary tissue, specifically on the various follicle and oocyte groupings. Eventually, the system would allow for potential investigations on ovarian protection from chemotherapy drugs such as doxorubicin.

CURRENT DEVICES

The *in vitro* culturing and maturation of tissues is a groundbreaking frontier with vast potential. The following is a summary of some of the devices currently used for culturing tissues or organs along with an in-depth description of the Langendorff heart. Finally, specific procedures are described for culturing follicles on varying biological scales, from culturing clusters of follicle cells to entire ovaries.

Tissue Bioreactors

The term bioreactor is not formally defined. However, as the term is used in tissue engineering, bioreactor can be defined as, "… any apparatus that attempts to mimic and reproduce physiological conditions in order to maintain and encourage cell culture for tissue regeneration" [8]. In all cases, cells need to be supplied with sufficient nutrition in order to proliferate. This is done with the flow of culture medium through the bioreactor and around the cells/tissue. Ideally, a bioreactor will meticulously control and monitor parameters that affect cell culture efficiency: pH, temperature, flow rate, mechanical stresses, biochemical gradients, etc. Bioreactors generally fall underneath two categories: rotating wall or fixed wall [8]. A rotating wall bioreactor involves an external wall that rotates producing a flow rate that will suspend the tissue in free-fall. A fixed wall bioreactor has stationary walls and the culture medium flow is controlled by other means including pumps or gravity [9]. Due to the complexity and variability involved in maintaining an environment suitable for cell or tissue culture, bioreactors specified for species and type of tissue are necessary.

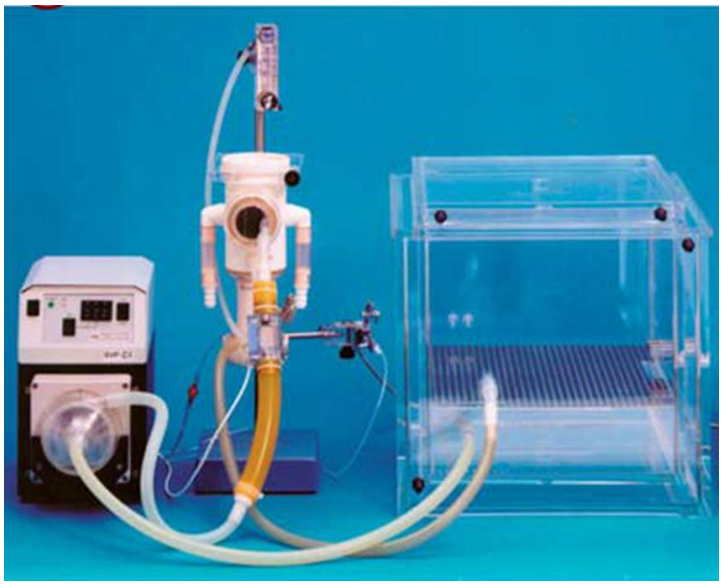

Figure 3. Example of bioreactor used for a pig kidney [10].

Bioreactors used for entire organ culturing are very similar to tissue bioreactors. Organ bioreactors have been successfully developed, though not for ovaries. Systems for isolating lungs, livers, and kidneys (**Figure 1**) of large animals are commercially available and have been shown to be highly beneficial for the research of how that organ responds to alterations of physiological variables [10].

Langendorff Heart

An organ bioreactor that was specifically studied in order to become familiar with *in vitro* maintenance of an organ was the Langendorff Heart. Extensive descriptions of the Langendorff Heart, as well as its uses and modifications, have been described [11]. Briefly, a heart is first extracted from a small animal (the concept can be used for animals of any size but smaller animals are typically used due to availability of hearts and equipment) and filled to diastole with a blood analog, such as the Krebs and Hensleit (KH) solution. For the left ventricle, a cannula is inserted into the aorta to supply pressure upon the aortic valve. This pressure causes the aortic valve to close, which results in KH solution being pumped to the coronary arteries, thus supplying the heart with nutrients. A cannula is also inserted into the atrium in order to fill the heart with the KH solution. As the heart pumps the fluid out of the aortic valve, a peristaltic pump retrieves the fluid, pumps it through an oxygenation chamber and then back into the left atrium. In this way, the left heart is maintained. Thanks to Dr. Tim Hacker, access to a Langendorff Heart model was obtained in order to see an example of how the vasculature of an organ can be replicated *in vitro.*

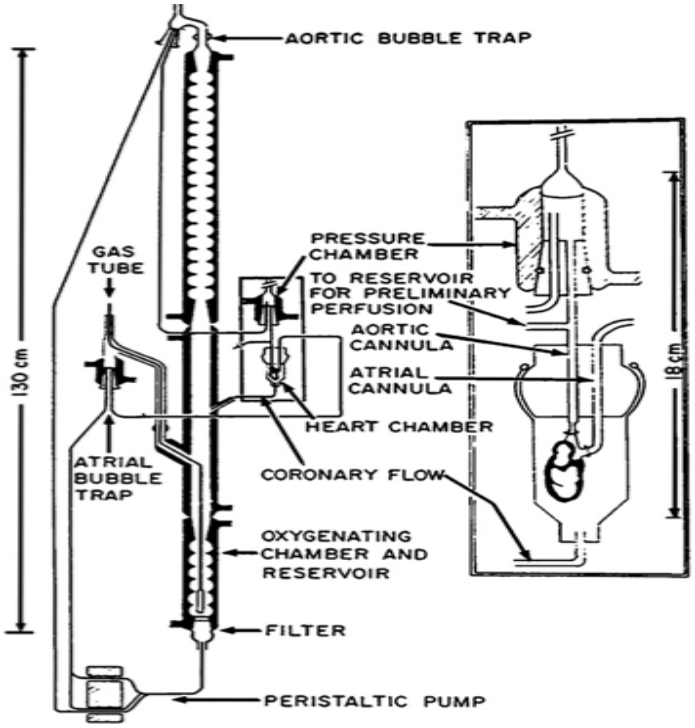

Figure 4. Isolated working heart model. Modification of the isolated heart perfusion model (Langendorff), which allows measurement of the left ventricle work [11].

Follicle Cell Cluster Culture

The culturing of follicle cell clusters began with two-dimensional (2D) techniques involving either multi-well plates or microdrop cultures [12]. These techniques were successful in sustaining the follicle for the duration of its maturation. It is now known that three-dimensional (3D) techniques of culturing follicles are much more efficient and physiologically relevant [13,14]. These techniques involve encapsulating the follicles in matrices that mimic the extracellular matrix (ECM) that the cells are exposed to *in vivo*. The types of matrices include but are not limited to alginate gels [15], matrigels [16], collagen gels [17], and agar hydrogels [18]. 3D culturing techniques are often considered superior to 2D techniques because they allow for the follicle to grow out in all directions and better simulate the ECM. However, the best technique for follicle cell culture is highly dependent on species, stage of development, and field of study.

Ovarian Culture

The culturing of ovarian tissue has been ineffective to date. Specifically, follicular development past the pre-antral stage is inhibited when culturing human ovarian biopsy tissue [13]. In humans, however, the culturing of ovarian biopsy tissue has been beneficial for developing the primordial follicles as far as the pre-antral stage, and then the follicles are removed to be cultured as follicle cell clusters to study antral stage development [19]. In this technique, the ovary tissue was cultured for a total of six days at which point the growing follicles were identified and extracted from the tissue for further study.

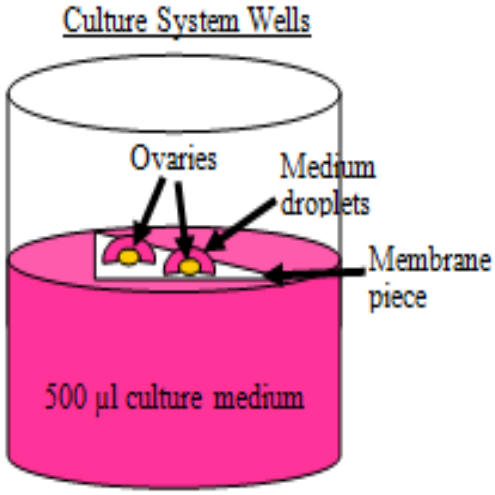

Figure 5. Isolation of neonatal ovaries and establishment of whole ovarian culture system [20].

The current method used by the client for culturing ovaries *in vitro* involves the extraction of neonatal mouse ovaries. A detailed protocol can be found elsewhere [20]. In short, the neonatal mouse ovaries are extracted from the mouse immediately after euthanasia. Each well of a 48-well plate is prepared with 500 µL of medium and a Millicell culture insert. The neonatal mouse ovary is then placed on the insert followed by a drop of medium on top of the ovary. The plate is kept in an incubator with the medium being changed every two days. Using this procedure, the neonatal mouse ovaries are able to live from 1-15 days. This procedure has also been used by O'Brien in order to culture ovaries, extract the follicles and eventually produce live offspring [21,22].

Although these culturing techniques have been beneficial, there has been no procedure developed that can culture complete, mature ovaries for an extended period of time.

DESIGN REQUIREMENTS

Before starting the design process, the bioreactor product design specifications were compiled (**Appendix A**). Most importantly, the bioreactor needed to provide an environment that would allow for the culture of cow ovaries/tissues *in vitro*. It was determined that a successful design would be able to sustain ovarian cell viability within a range of 90-100%. The size of the bioreactor will depend on the biological scale chosen for the culture. The bioreactor must operate in an incubator that provides an environment of 37˚C and 5% CO_2. The bioreactor must be easily sterilized so that it can be used in a biological safety cabinet (BSC) and provide the ovary with a sterile environment. The proposed length of culture in which the bioreactor must function is a minimum of 2 weeks, with a 3 month maximum. The bioreactor should be easy to use and durable so that it can be operated for up to 5 years with little variation between experiments. Precise monitoring and adjustment of media flow rates (≤30 mL/min)[22], pressure (≤203 mmHg)[23], temperatures (≤40˚C), and pH (6-8) should be possible (**Appendix A**). Inflow of therapy agents or hormones at the discretion of the user should also be possible. The cost of the

final device should be as inexpensive as possible, with a maximum of $3,000 for materials and assembly.

DESIGN ALTERNATIVES

Design Process

Two separate considerations were made during the overall design process: biological scale and bioreactor technique. The first decision was determining the biological scale of culturing the ovary. Different biological scales were compared based on plausibility and relevance to the problem statement. The second decision involved researching the biological scale chosen and then developing a design for a bioreactor technique. For each decision, three design alternatives were proposed. The design alternatives were evaluated and given a score in relevant categories weighted based on importance. The design alternatives were then compared in a design matrix, and the design that scored the highest was pursued further.

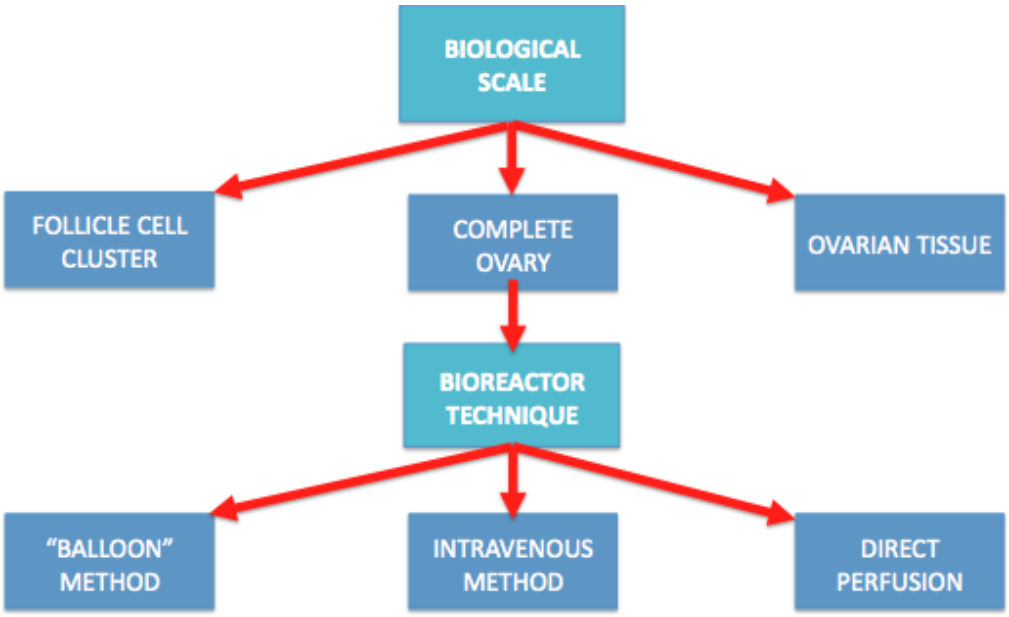

Figure 6. Flow diagram of design process. First biological scale was considered and then bioreactor technique was considered.

Biological Scale

Due to complexity of culturing complete ovaries, multiple biological scales were assessed. These biological scales were follicle cell cluster, ovarian tissue, and complete ovary.

Design Alternative 1: Follicle Cluster

Primordial follicles are essential for fertility preservation because they are the most abundant follicle type, are present in females of all ages, can resist different types of chemotherapy, and can withstand cryopreservation and thawing [14]. Successful *in vitro* culture of primordial follicles has been limited by the large complexity of the cell-cell and cell-stroma interactions that are needed for growth [24]. Consequently, optimal culture

techniques are 3D *in vitro* culture systems that effectively imitate the ovary's internal architecture, as opposed to more simplistic 2D culture systems [14,13,24,25].

Various 3D *in vitro* culture systems have been developed. When designing 3D culture systems, the chemical and physical properties of the biomaterial must been extensively critiqued. Properties such as elasticity, rigidity, and diffusion are of particular importance [13]. Current models for 3D culture include follicle cell cluster encapsulation in a matrix, suspension culture, serial culture, and microfluidic culture. Each technique offers both advantages and disadvantages.

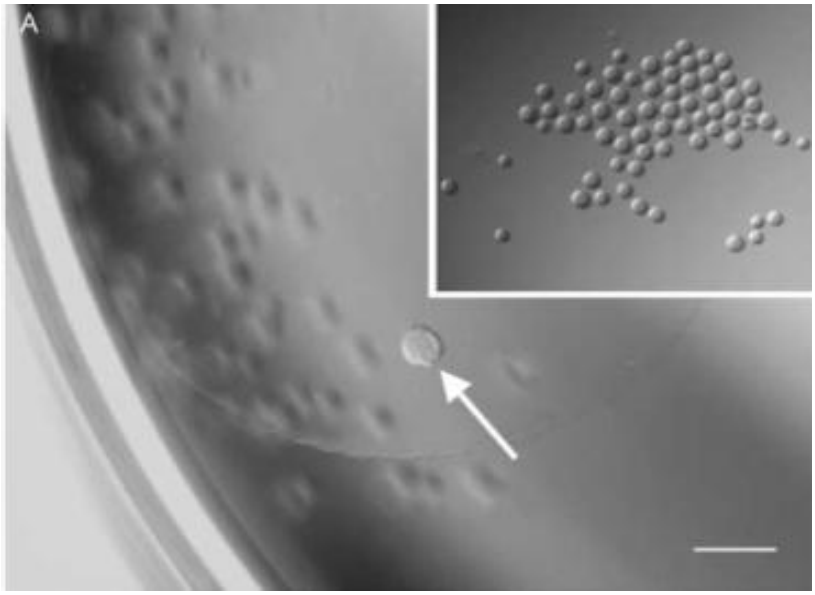

Figure 7. Representative image of follicle in alginate hydrogel bead in culture well with oocytes [25].

Studying the growth and maturation of the primordial follicle using follicle clusters offers some distinct advantages. First, ovarian follicle culture has been attempted and successfully achieved on wide scale in tissue engineering. Furthermore, there have been numerous publications outlining a clear method and procedure to accomplish follicle culture. This makes this design alternative extremely feasible. The availability of follicles makes this biological scale appealing as well. The option to extract many follicles from a single source is a large advantage.

There are several disadvantages to follicle culture. The lack of a natural extracellular matrix (ECM) does not allow the study of cell-cell and cell-stroma interactions of follicle cells in their natural environment – the ovary. Because only the follicle is cultured and studied, there is little clinical and physiological relevance for this alternative. Additionally, this will limit the possibility for future testing in the Salih Laboratory.

Design Alternative 2: Ovarian Tissue

Research has shown that extracellular matrix (ECM) is very advantageous in ovarian cultures, due to its ability to maintain the three-dimensional organization of the follicle [26]. Using natural ECM has also shown to significantly increase the viability of follicles and

increase the growth of many other cell types [27]. The desire to study follicles in their natural environment, while limiting the complexity of the design led to the second design consideration – ovarian tissue. The culture of human ovarian cortical tissue has been achieved using either slices or cubes of tissue [28].

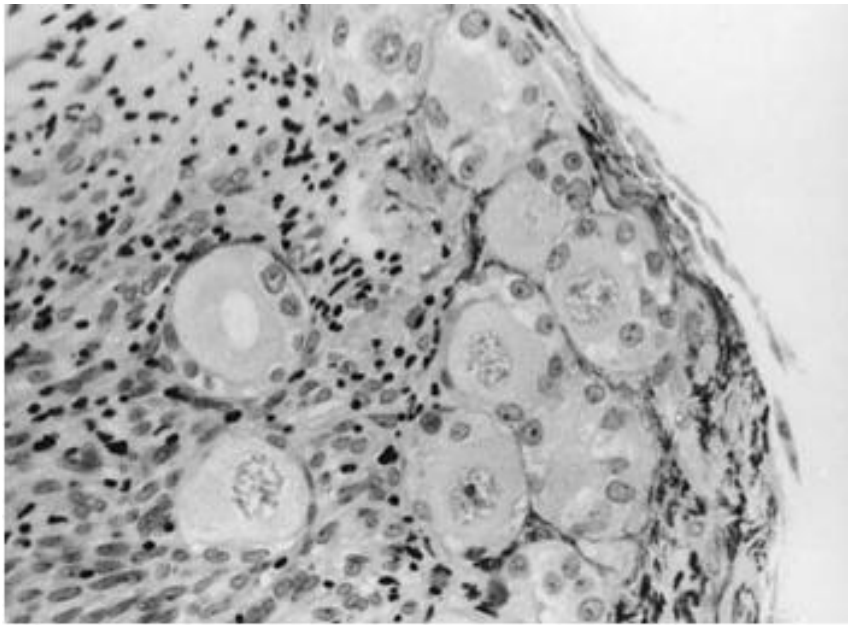

Figure 8. A cluster of follicles in ovarian tissue slices cultured for 7 days in Earle's solution on extracellular matrix; original magnification X400 [28].

For this project, thin slices of the outer layer of the ovary would be cultured. No studies have been performed to identify optimal tissue dimension or surface area to volume ratio for culturing early stage follicles [28]. In order to make the design less complex, small sections of ovarian tissue will be cultured.

Design Alterative 3: Complete Ovary

The third design alternative for the biological scale was the complete ovary. Neonatal ovarian cultures are well suited for studying follicle formation [29,30], the role of growth factors in follicle growth activation [12], and the effect of chemicals on follicle populations [31]. Both neonatal rat and mouse ovaries have been cultured, allowing ovarian development and physiology to be studied. However, using current methods, ovaries can only be cultured for 1-15 days [20].

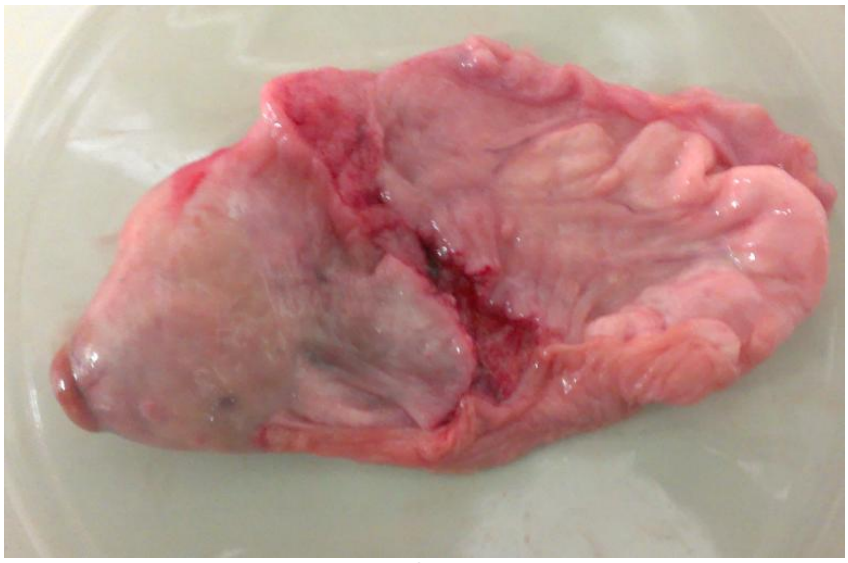

Figure 9. Image of mature cow ovary.

For this design, a complete cow ovary will be utilized. This type of mammalian ovary is large in size – on average 35x25x15 mm – and has more pronounced features than rodent ovaries [32]. Additionally, the vasculature of cow ovaries is easily accessible and has been extensively studied [33,34]. This is a significant advantage. Furthermore, by using a complete ovary, the follicle cells and the surrounding tissue can be studied in physiologically accurate conditions. This leads to an extremely high clinical relevance. It must be noted that extended mature ovary culture has not been achieved.

BIOLOGICAL DESIGN MATRIX

Table 1. Biological scale design matrix.

Biological Scale				
Factors	**Weight**	**Follicle Cluster**	**Ovarian Tissue**	**Complete Ovary**
Feasibility	30	27	23	22
Clinical Relevance	30	18	22	30
Ease of Culturing	20	18	15	15
Consistency	15	12	10	15
Cost	5	3	4	5
TOTAL POINTS	**100**	**78**	**74**	**87**

Feasibility

Feasibility is if the design alternatives can be grown or cultured, and how difficult it would be to culture. This was the first factor that was considered in the design and has chief important. Consequently, feasibility was weighted highest (30). Based on research and previous studies, the feasibility of follicle cell cluster, ovarian tissue, and complete ovary culture was assessed. Follicle culture has been successfully achieved, giving it a very high feasibility score of 27. Ovarian tissue culture has also been accomplished, but only as a method to grow follicle cell and only for a short period of time. Therefore, ovarian tissue received a feasibility score of 23. Extended ovary culture has not been accomplished; however, short-term ovary culture is currently performed and there methods for culturing other organs and tissues. This led to the complete ovary receiving a score of 22.

Clinical Relevance

Each biological scale design alternative was assessed on its relevance to the client's research. This was based on physiological relevance and the ability to test chemotherapy toxicity and protection. Follicle clusters are not representative of the entire ovary; however, they are the base unit of the female reproductive biology. This led to a score of 18, the lowest clinical relevance score. Similarly, ovarian tissue is not representative of an entire ovary, but it does have a natural ECM. This led to a score of 22. The complete ovary scored a 30, as it is exactly what needs to be studied.

Ease of Culturing

Ease of culturing is the simplicity, or complexity, in day-to-day actions needed to culture the different design alternatives. As the goal of the project is culture the ovary, this factor was given a high weight (20). Due to the fact that follicle culturing has already been achieved, and a procedure to achieve this is available, this design alternative scored the highest with 18 points. Ovarian tissue has also been successfully culture, but there is a much higher degree of difficulty when culturing for an extended period of time. Thus, it received a score of 15. Complete ovaries would also be difficult and would require similar culturing procedures as ovarian tissue (i.e. media volumes, media changing periods, and environment). Consequently, complete ovary scored a 15.

Consistency

Consistency is the similarity from batch-to-batch of each design alternative. As this is a crucial factor in order to create reproducible and precise results, it received a weight of 15. Follicles would be provided from ovarian samples, and if multiple samples are used there can variation between follicles. Thus, follicle cluster scored a 12. Multiple ovarian tissue samples can be taken from a single ovary, leading to increased consistency. However, follicle clusters are located sporadically around the ovary, causing varying follicle density in each ovarian tissue slice. These aspects led to a score of 10. Complete ovaries would be purchased in bulk and will all have similar size, vasculature, and properties. Complete ovaries therefore received the highest score of 15.

Cost

The cost of purchasing the follicles, ovarian tissue, or cow ovaries must be considered, as the cost of the design must be kept to a minimum. However, the client has expressed great

willingness to invest in the project. This gave cost a weight of only 5. Follicles are the most expensive, as they must be carefully removed from ovaries, leading to a score of 3. Complete ovaries are purchased in bulk at lower prices, giving it a score of 5. Ovarian tissue can be cut from complete ovaries; however, this requires time and precise tools. Thus ovarian tissue received a score of 3.

BIOREACTOR/CULTURING TECHNIQUE

After considering the biological scale, a bioreactor and culturing technique was developed for the highest ranked biological scale – complete ovary. After research and extensive discussion, it was determined that three design alternatives for the bioreactor and culturing technique need to be significantly assessed and considered. The three design alternatives selected were the "balloon" method, intravenous method, and direct perfusion method.

Design Alternative 1: "Balloon" Method

The first proposed design alternative was the "balloon" method. It is known that the supply of oxygen and soluble nutrients is a significant limitation in culturing 3D tissues *in vitro* [35]. This alternative inserts a metal/plastic chamber as a novel method to provide nutrients to the interior of the ovary (**Figure**), addressing these limitations. The interior chamber has a single input tube at its top and multiple output tubes located at the sides and bottom of the sphere (**Figure**). These will facilitate media flow across the interior of the ovary in a uniform manner. A pump would be utilized to regulate and control media flow. In order to have the ovary fit around the interior chamber, the inside of the ovary will be removed. Theoretically, this alteration to the ovary will not affect testing and viability, as the follicles are located on the outside tissue of the ovary.

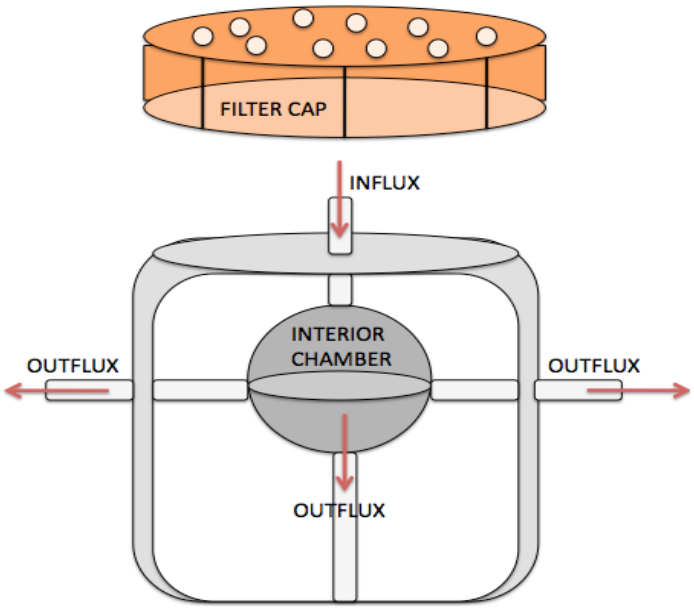

Figure 10. Conceptual diagram of the "balloon" method.

The entire interior chamber and ovary would then be placed in a large container. The container would then be filled with additional media in order to provide the exterior of the ovary with nutrients. The entire system would then be sealed using a filter cap. The filter cap would allow for necessary diffusion of gases. All components involved in the bioreactor will be sterilizeable, either by autoclave or by soaking in ethanol solution.

Design Alternative 2: Intravenous Method

The second design discussed as a culturing technique was to utilize the existing vasculature of the ovary to deliver nutrients to the follicles. Cannulas inserted into the ovarian artery in order to flow media to the follicles gave this design its name: the intravenous (IV) method. To address the issue of follicular nutrition, this method was based around using the existing, fully functional nutrient delivery system of the cow's ovary. The basic mapping of the ovarian arterial vasculature can be seen in **Figure.** Tapping into the vasculature would allow for direct and effective transportation of media to the vital areas of the ovary, like the follicles.

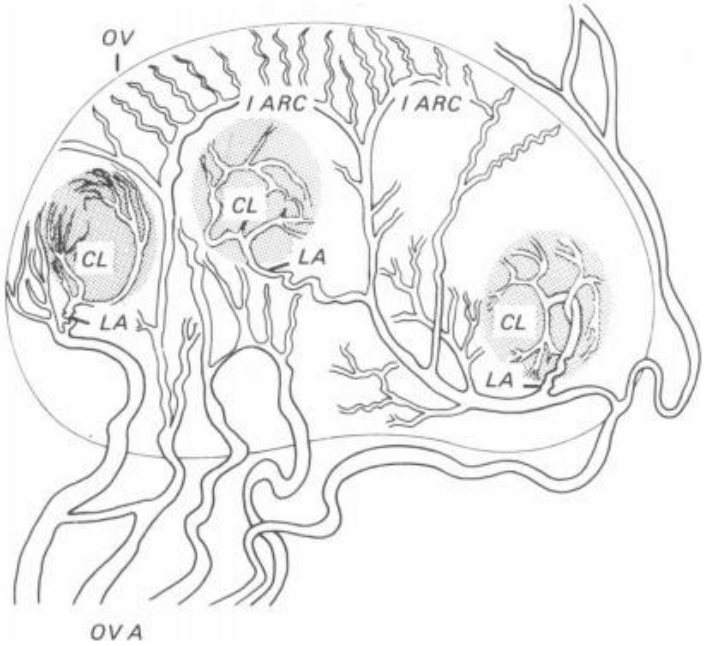

Figure 11. Diagrammatic representation of the arteries to the ovary and corpora lutea of the guinea-pig [36].

For this design to work effectively, it must mimic the *in vivo* conditions of the ovary as closely as possible. The primary components and the flow of media in the IV method are depicted in the block-diagram below (**Figure 2**)**.** The housing for the ovary will be a 250 mL Pyrex bottle with two ports in the cap for inflow and outflow tubing to transport media. The ovary will rest on a platform with a porous membrane for diffusion of media into the follicles, supplementing the media being supplied to the ovarian artery. The platform will be attached to the cap of the bottle to enable the ovary to be easily taken in and out of the bioreactor. All components involved in the bioreactor will be sterilizeable, either by autoclave or by soaking in ethanol solution.

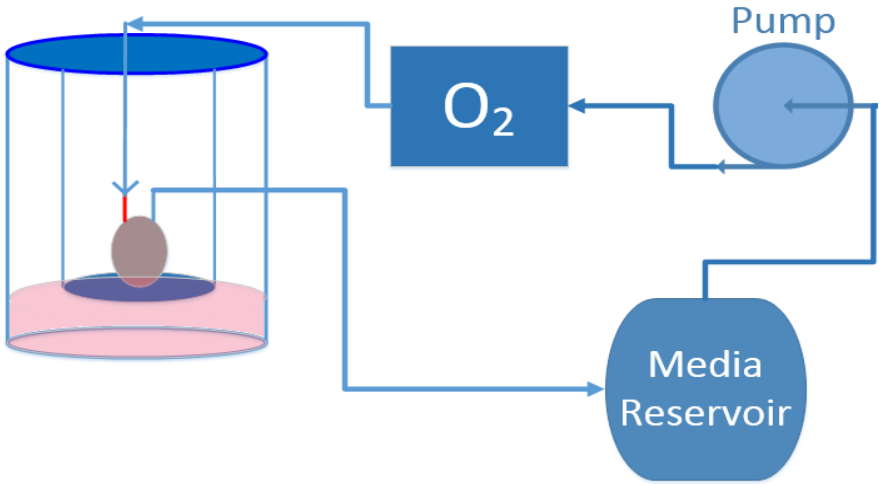

Figure 12. Conceptual diagram of the intravenous method for ovarian culture.

The second component of the IV design is the media. The media will be transported from the reservoir through tubing to the ovary by a pump. Since ovarian blood flow is more steady than pulsatile *in vivo* [22], the pump can provide media to the ovaries at a constant flow. The media also needs to be oxygenated to avoid hypoxic conditions in the follicles. This will be addressed by flowing the media through an oxygenator prior to entering the bioreactor and the ovary. The tubing will be attached to the ovarian artery by cannula, allowing the media to enter the vasculature.

Design Alterative 3: Direct Perfusion

It has been shown that static culture conditions result in poor cell migration and only cells around the exterior of the scaffold/tissue are viable instead of the desired, uniform 3D culture [37,38]. The supply of oxygen and soluble nutrients has also been a limiting factor for the *in vitro* culture of 3D tissue [35]. This can be overcome by a perfusion system. In these systems, medium flows unidirectionally through the pores of a scaffold (**Figure).** The flow of medium directly through the scaffold or tissue being cultured enhances nutrient transport to the interior of the scaffold/tissue and provides mechanical stimulation in the form of liquid shear [9]. Such direct perfusion bioreactors have been used in tissue engineering extensively.

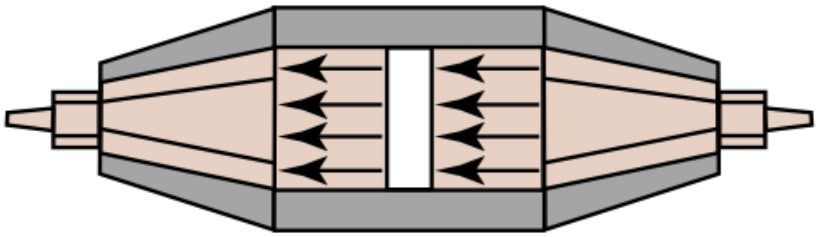

Figure 13. Example of a direct perfusion bioreactor in which the medium flows directly through the scaffold [35].

Due to their wide success and simplicity, direct perfusion bioreactors were considered as the third design alternative. In this design, the scaffold being cultured would be a complete ovary. In order to increase mass transfer and diffusion of oxygen and nutrients across the

entire ovary, the interior of the ovary will be removed. The media will be pumped through the bioreactor and must be oxygenated to avoid hypoxic conditions in the follicles. This will be addressed by flowing the media through an oxygenator prior to entering the bioreactor and the ovary. This will allow approximately equal and even distribution of nutrients to all parts of the ovary. However, the effects of direct perfusion are highly dependent on medium flow-rate, and optimal conditions can be challenging to determine [35]. All components involved in the bioreactor will be sterilizeable, either by autoclave or by soaking in ethanol solution.

BIOREACTOR/CULTURING TECHNIQUE DESIGN MATRIX

Table 2. Bioreactor/culturing technique design matrix.

Bioreactor/Culturing Technique				
Factors	**Weight**	**"Balloon" Method**	**Intravenous Method**	**Direct Perfusion Method**
Cell Viability	20	15	18	10
Physiological Accuracy	20	15	20	13
Ease of Use	15	13	12	14
Biocompatibility	15	14	14	14
Repeatability	10	7	9	8
Versatility	10	6	8	3
Cost	5	3	2	4
Ease of Assembly	5	2	2	4
TOTAL POINTS	**100**	**75**	**85**	**70**

Cell Viability

The ultimate goal of the design is to successfully culture and grow ovaries outside the body, so cell viability of the ovarian tissue is crucial. Thus cell viability was given a weight of 20. The "balloon" method provides nutrients to the inside and outside of the ovarian at the same time leading to increased viability. The intravenous (IV) method also does this; however, it uses the vasculature and provides nutrients in a physiologically accurate manner. Consequently, the "balloon" method received a score of 15 and the IV method received a score of 18. Supplying nutrient by direct perfusion utilizes diffusion and many cells in the interior of the ovary may not be given enough nutrient. Hence, it received a score of 10.

Physiological Accuracy

Physiological accuracy is how precisely the bioreactor/culturing technique is able to create conditions and supply the ovary with nutrients in a manner that mimics the body. This is a very significant consideration in the design and was given a weight of 30. The ability to effectively create physiological conditions directly correlates to the ability to successful culture. Both the "balloon" method and direct perfusion method use flow and submersion to provide nutrients. The "balloon" method adds nutrients directly to the interior of the ovary, while the direct perfusion method does not. This led to a score of 15 for the "balloon" method and a score of 13 for the direct perfusion method. The IV method provides nutrients through the ovary's vasculature, which is how nutrients are provided in physiological conditions. Thus, the IV method received a 20.

Ease of Use

This factor rates the ergonomics and how easily the user can operate of the bioreactor system. This factored received a weight of 15, as it is essential that many different members of the Salih Laboratory can use the device and to limit preparation time for the culturing procedure. Both the "balloon" and IV method require manipulation of the ovary. In the balloon method, the interior of the ovary must be removed and in the IV method, the arteries and veins must be found and cannulized. This lows ease of use, giving the "balloon" method and IV method scores of 13 and 12, respectively. The direct perfusion method is very simplistic and the ovary only needs to be placed in the bioreactor system. Thus, it received a score of 14.

Biocompatibility

Biocompatibility was considered for the bioreactor technique, as the bioreactor will be in significant contact with cells and living tissue. Thus, all elements of the bioreactor need to be non-cytotoxic and compliant with cell culturing. All design alternatives scored a 14, as all will be significantly biocompatible and will use similar materials.

Repeatability

This factor assesses the bioreactor techniques capability to reproduce the same culturing conditions for each experiment. Repeatability was given a weight of 10. Due to the fact that the IV method flows media through the vasculature, nutrients is provided is the same manner each time. Thus, it received a score of 9. The direct perfusion method flows media at a constant rate across the entire ovary; however, the rate of diffusion of the media can be varying. This in addition to the removal of the interior of the ovary can cause differences between trials. Consequently, the direct perfusion method received a score of 8. The "balloon" method scored the lowest value (7) because the interior of the ovary is removed and the absorption of media is non-uniform across the entire ovary.

Versatility

This factor rates the design alternatives ability to manipulate and change the environmental conditions of the bioreactor. Some of these conditions include flow rate, pressure, growth factors, and media types. This is important, as it will allow for wide range of testing to be performed. It also assesses the ability of the bioreactor system to be used for different ovary types (i.e. cow vs. human). This is an important consideration because

the ultimate goal of the client is to achieve human ovary culture. Overall, this factor does not have significant weight at this point in the design process, and was given a weight of 10. All methods have the ability to alter flow rates; however, only the "balloon" and IV method can alter interior flow. This greatly increases versatility. However, because the inside of the ovary is removed in the "balloon" method, this versatility is severely hampered. The IV method has the ability to change flow rate, pressure, and growth factor by using the ovary's vasculature, thus achieving change in a physiologically accurate way. This lead to the following scoring: "balloon" method – 6, IV method – 8, and direct perfusion method – 3.

Cost

The cost of purchasing the components of the bioreactor/culturing system must be considered, as the cost of the design must be kept to a minimum. However, the client has expressed great willingness to invest in the project. This gave cost a weight of only 5. Both the "balloon" and IV methods require many components and very accurate pumps, as there is interior flow. This dramatically raises the cost. This gives the "balloon" method a score of 3. The IV method also needs medical equipment (i.e. cannulas) to function. This gives the IV method a low score of 2. On the other hand, the direct perfusion bioreactor is a market device and can be easily purchased. It also requires no additional component. Consequent, the direct perfusion method received a score of 4.

Ease of Assembly

This factor assesses the difficulty of putting together the individual parts of the bioreactor to make a complete, functioning system. It also considers any alterations to the ovary that are required for the design. The direct perfusion method would only require the interior of the ovary to be removed and then the ovary to be place in the bioreactor. The bioreactor has a simple set-up and thus was given a score of 4. Both the "balloon" and IV method involve more complete modifications to the ovary prior to use and have multiple flow tubes. This leads to more complex systems and assemblies, and both alternatives receiving a score of 2.

FINAL DESIGN

Final Design Selection

The final design incorporates the strongest selections from the two design matrices: the ovary on the biological scale and the IV method for the culture method. The complete ovary was chosen because it scored highest in the clinical relevance, repeatability, and cost categories of the biological scale design matrix. The IV method received the highest marks in the top two categories of the bioreactor/culture method design matrix: cell viability and physiological accuracy. The ovary used for this design will be the cow ovary, due to the ease of access to the vasculature and high clinical applicability and relevance to human ovaries.

Final Design Specifications

Most of the constituents (bottle, cap, pump, and oxygenator) selected for the final design are commercially available products. This eliminates the need to make custom parts and

also utilizes validated parts in the design. The bioreactor design will be comprised of two main components, the cap assembly (**Figure 3**) and the bottle (**Figure 4**). The block diagram, as seen in **Figure 2**, outlines the flow of media from start to finish.

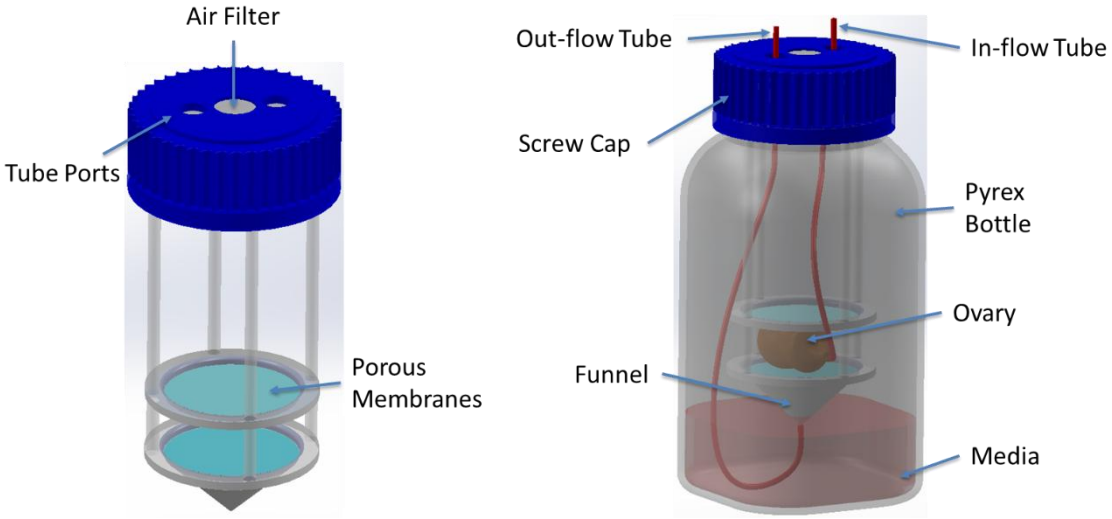

Figure 14. Solidworks rendering of removable cap apparatus (left) and assembled bioreactor (right).

In the cap assembly, the ovary will rest between two plates with porous membranes to allow diffusion of media into the follicles. The plates will be 40 mm in diameter to account for the average length of the ovary (35 mm) [13]. The bottom plate has a funnel connected to its underside to allow for media to be taken back to the reservoir. The top plate will slide up and down along rods and will hold the ovary in place. The rods will be screwed into the bottom of the cap, making the cap assembly one connected piece.

The cap will be an Omnifit "T" series cap (00945T-2F) with a 45 mm diameter and two ports: one for inflow and another for outflow tubing. The media flow ports will have barbed connectors on the top of the cap to connect the tubing to and from the outside circulation. The cap assembly will connect the tubing from the pump and media source to the ovary with two sections of tubing each for the inflow and outflow. For inflow, one section of tubing connects from the media reservoir to the port on the cap, and the other goes from the inside of the cap to the ovarian artery. For outflow, one section of tubing connects the bottom of the funnel to the inside of the cap, and the other section runs from the port on the cap to the media reservoir. The barbed connectors permit separate attachment of the tubing from the outside media circulation. Thus, the cap assembly component allows external set-up, as well as facilitates easy transfer of the ovary into and out of the bioreactor.

The inflow tube will be attached to the ovarian artery through the use of a luer stem, which can be inserted into the artery and tied off with nylon to provide a sealed connection. To remove media from the bioreactor, the bottom platform funnels excess media to the outflow tube. This design was chosen over connecting the tube to the ovarian vein because, as seen in the Langendorff heart, the media will not have enough pressure to flow entirely

through the vein and will instead diffuse out of the organ in many places. Allowing the media to funnel into the outflow tubing will ensure all of the media is pumped back to the reservoir.

As mentioned earlier, the housing for the ovary will be a 250mL Pyrex bottle. The bottle and cap can be autoclaved, maintaining the sterility requirement. Media will be filled up to the bottom of the ovary, covering it roughly 2mm. This will keep the ovary moist and will promote media diffusion into the follicles.

FUTURE WORK

Current Semester

The two main goals for the spring 2013 semester are to construct the bioreactor system and to begin cell viability testing. With the primary source of failure for ovary culture being low cell viability, the foremost objective will be improving cell viability. To do this, the bioreactor will be composed mainly of specialized, commercially available parts so the system can be assembled and tested as quickly as possible. This will allow for more time to improve the process of culturing the ovary.

For the bioreactor system, the Pyrex bottle, Omnifit cap, tubing, cannulas, porous membranes, oxygenator, and pump will be purchased, while the platforms, funnel, and rods may have to be separately designed and machined. The exact dimensions and model numbers of the tubing, cannulas, oxygenator, and pump have not been decided at this time. The platforms and rods of the cap assembly will be made of Polyamide-12 and generated by a rapid prototyping machine. Polyamide-12 was chosen due to its low water absorption, resistance to cracking, and its ability to be autoclaved (max short term temperature of 140°C) [39].

To assess follicle viability, small biopsies can be taken periodically and tested by live/dead staining to get a rough idea of how viable the organ is. Testing various hormone concentrations, like follicle-stimulating hormone (FSH), can provide information on the current stage of the follicle transition. A more extensive test, sectioning and immunostaining, will be performed once experimentation has been completed to accurately determine overall viability of the ovary.

Future Semesters

In future semesters, the primary goal is to integrate the bioreactor with the DXR Chemobag project currently being developed by another BME design team at the University of Wisconsin-Madison [40]. The Chemobag is designed to release known amounts of the DXR agent, which will then be absorbed by the ovary and lead to ovarian insult. The degree and extent of the insult can then be determined, with the ultimate aim being to create preventative measures. Once both projects are at a more advanced stage, the integration process will begin.

Another objective in future semesters is to digitally monitor the conditions of the bioreactor, like temperature, pH, hormone concentrations, and DXR levels. This information will provide real-time conditional data and can help determine exactly when and why potentially harmful changes occur. This diagnostic tool will facilitate the culturing efficacy of the bioreactor and result in a more complete research platform.

ACKNOWLEDGEMENTS

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APPENDIX A: PRODUCT DESIGN SPECIFICATIONS

Novel in Vitro Model to Grow and Culture the Culture the Ovaries Outside the Body
Product Design Specifications
March 6th, 2013

Client: *Dr. Sana M. Salih, MD, MMS*

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Function:
Oftentimes female patients lose their reproductive capabilities as a consequence of having cancer or from undergoing cancer chemotherapy. Research has shown that doxorubicin (DXR) chemotherapy causes ovarian insult, ultimately leading to ovarian failure. Currently, no systems exist to grow adult ovaries in vitro to test chemotherapy toxicity and protection. This situation greatly limits ovarian research. A system to test the effects of the chemotherapy agent DXR on the ovaries needs to be developed. This project will establish a novel ex-vivo ovary culture system to maintain cell viability in order to facilitate assessment of chemotherapy toxicity and protection. The device will utilize the ovaries vasculature, providing the ovaries with necessary nutrition in a physiologically accurate manner to prevent necrosis. The fluid flow rates and pressure of the infusion lines will be adjusted to physiological conditions to ensure ovarian health. The ovary scaffold will be contained within a sealed environment capable of maintaining internal sterility and cell viability.

Client Requirements:
- Establish novel technique to grow ovaries outside the body
- A method that mimics ovarian vasculature must be developed to properly deliver nutrients to the ovary in a physiologically accurate manner
- Bioreactor must provide sufficient nutrients to facilitate the transition from primordial follicle to primary follicle and prevent necrosis
- Fluid flow rate and pressure must be accurately monitored using digital controls to ensure optimal ovarian health
- All components of the bioreactor must be biocompatible and non-toxic
- Bioreactor must be durable and be able to function for experiments last up to 3 months
- Price of device should be as low as possible

Design Requirements:

1. **Physical and Operation Characteristics**

a. *Performance Requirements*: This project will involve culturing ovarian tissues/ovaries. The bioreactor will culture individual cow ovaries (35x25x15mm in size) [13]. The bioreactor will need to maintain significantly high cell viability (~90% – 100% viability) and sterility for ovarian follicle cells. Bioreactor must provide sufficient nutrients to facilitate the transition from primordial follicle to primary follicle and prevent necrosis. Infusing media in the ovarian artery, or creating an artificial circulation system through the ovary will achieve this. The ovary bioreactor device needs to accurately detect and measure fluid flow rate and pressure when ovaries are present and when they are not. Flow rate must approximately 30 mL/min [22] and blood pressure should be approximately 203/189 mmHg [23]. The device will have the ability to be used multiple times, for multiple experiments with experiments lasting up to 2 weeks. All opening to the bioreactor must be either sealed or outfitted with a filter to maintain sterility

a. *Safety*: All materials in the device must be safe for handling under basic laboratory safety procedures and National Institute of Health (NIH) protocols. The device should be in compliance with mammalian cell culture standard operating procedures. The bioreactor must create an enclosed environment to ensure all materials/fluids that contact cells are secured and do not contact user.

b. *Accuracy and Reliability*: The bioreactor must be able sustain ovary follicle cell viability for 2 weeks at approximately 90%. To assess ovarian tissue viability, H2W staining of ovarian slices and TUNEL assays will be performed. Additionally, this device will need a great deal of precision (repeatability) and accuracy in creating and monitoring of fluid flow and pressure similar to physiological values, as this can greatly affect cell culturing.

c. *Life in Service*: The bioreactor device will need to support cell viability and function accurately for approximately 2 weeks for short term testing. Long term testing will require the device to maintain ovary tissue viability for approximately 3 months. This will provide adequate time to facilitate the transition from primordial follicle to primary follicle.

d. *Shelf Life*: The device should be able to function accurately for approximately five years, so that it can be used for a multitude of experiments and stored for future use. Once in use, the device must persist and maintain accurate functionality throughout an entire experiment and work effectively in the presence of cell culture media and cells.

e. *Operating Environment*: The ovary bioreactor will be used in an incubator to create an environment (37°C and 5% CO_2) that mimics facets of the in-vivo environment of ovary follicle cells and bathed in standard cell culture media (Ham's F-12/DMEM). For cell culturing, the bioreactor will need to be easily sterilized using ethanol, or an autoclave, so that it can be used in a biological safety cabinet (BSC). The inside of the bioreactor should create a sealed, sterile environment for cell culture.

f. *Ergonomics*: The ovary bioreactor device should be easy to use, in order to ensure a high level of repeatability in experiments among different users. The device should be able to be used with limited experience, as well as by different and multiple users.

g. *Size*: The size of the ovary bioreactor device should be slightly larger than a cow ovary (35x25x15 mm) at least 45x35x25 mm in size. This will eliminate the potential problem of varying ovary size. In order to be housed within an incubator, the bioreactor and other device components should all fit within an incubator, occupying limited size; however, the pump for the device may be placed outside the incubator.

h. *Weight*: The weight of the device should be kept to a minimum in order to maximize ease of use and efficiency; however, weight is not critical in this design and is a low priority consideration.

i. *Materials*: The materials of the device should have no negative effects on cells and need to be non-cytotoxic. Specifically, the material used to mount the ovary should promote cell adhesion and not limit or inhibit the direction/orientation of ovary follicle cell growth. These factors must also be applied to the fluid flowing through the device. Additionally, the infusion lines mimicking ovary capillaries and vasculature should be small in diameter and create a sealed environment for cell culture media to flow through. If the media will be simply diffused to the ovary, the biocompatible and permeable membrane must be used to transport cell culture media.

j. *Aesthetics, Appearance, and Finish*: Ideally, the device should be clear and transparent, in order to allow the user to properly observe the ovaries being cultured. The entire bioreactor will be a hollow cylinder that will stand vertically on its axis. Aesthetics and appearance are not critical in this design and are a low priority consideration.

2. Production Characteristics

a. *Quantity*: There should be one ovary bioreactor device capable of housing one ovary.

b. *Target Product Cost*: The cost of the device should be kept to a minimum (below $3000); however, if a novel and repeatable method is developed, a higher product cost will be considered. Additionally, the client has shown great willingness to invest in the project.

3. Miscellaneous

a. *Standards and Specifications*: This device is not drug related, and therefore does not need approval by the FDA for use or testing. However, animal, and eventually human ovary, will be tested using this device so standard mammalian cell culture protocols and protocols must be adhered to. Additionally, all National Institute of Health (NIH) cell culture and biological safety protocols must also be adhered to.

b. *Customer:* The device is created for Dr. Sana M. Salih, MD, MMS. The overall goal of Dr. Salih's laboratory is to identify novel mechanisms to improve fertility and reproductive health in women. Specifically, fertility preservation in cancer patients. The device should be easy to use so that other members of the Salih Laboratory can use it. The highest priority for the customer is ensuring ovary viability over an extended 3-month period of time.

c. *Patient-related Concerns*: The device will be used with ovarian follicle cells and will thus need to be sterile for all uses. There are no concerns regarding data storage or confidentiality with this device, as the subjects are not patients.

d. *Competition*: Currently, no systems exist to grow adult ovaries in vitro to test chemotherapy toxicity and protection. However, there are methods used for follicle cell culturing and ovarian tissue culturing [13]. For follicle cell culture, methods include matrix encapsulation, suspension culture in rotating systems, serial culture, and microfluidic culture [28]. Organ bioreactors have been successfully developed, though not for ovaries. Systems for isolating lungs, livers, and kidneys of large animals are commercially available and have been shown to be highly beneficial for the research of how that organ responds to alterations of physiological variables [10].