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Production of bioactives using in vitro culture of cells from organs sourced from the meat processing industry

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Abstract

The production of bioactives using *in vitro* culture of cells from organs sourced from the meat processing industry provides a unique value-adding process. This project aimed to achieve proof-of-concept for a new technology for the amplification of target bioactive compounds from cells isolated from animal organs.

This project demonstrated the ability to disaggregate and isolate cells from two organs (corpus luteum and liver) of sheep and maintain the viability of these cells for more than one week. The addition of stimulatory factors to the culture medium resulted in the amplification of target bioactives up to 20-fold in liver cells and 43-fold in luteal cells. Furthermore, the amplification of two valuable target bioactives was achieved with a single stimulus in liver cells, demonstrating multiple bioactives can be induced simultaneously. This project achieved its stated aims within the specified timeframe. As a result we are ready to commence the next phase. It is recommended that the next phase of this project directs focus towards a marketable target bioactive with a quick route to market for commercialisation of this technology.

Executive Summary

Valuable bioactive products have been extracted from animal organs for many years. This is a one-off process that results in low level yields of product with associated expensive purification costs. Alternatively, genetically modified recombinant technology for the generation of bioactives can be used. This process is highly reproducible and scalable but has a high capital cost in the growth of cells for the production of the bioactive product and in meeting regulatory requirements.

This project was designed to demonstrate a proof-of-concept of the feasibility of the amplification of bioactives from primary cells derived from farmed animal organs. The advantage of using this approach is the production of target bioactives at high yields in a highly reproducible system with low purification cost. Additionally, this concept uses relatively low cost raw materials, involves relatively low production costs and allows for the production of multiple bioactives in a unitary process.

The aims of this project were to demonstrate a proof-of-concept of bioactive production from primary cells derived from farmed animal organs by:

- Demonstration of the successful disaggregation of two organs to the specific tissue cell types
- Demonstration of the ability to culture isolated liver and luteal cells *in vitro*
- Development of analytical methods for the detection and quantification of target bioactives
- Demonstration of the ability to amplify more than two bioactive products from *in vitro* cultured cells isolated from animal organs
- Demonstration of the scalability of the process from small laboratory scale plates to large tissue culture flasks
- Development of an integrated process from organ collection through to bioactive amplification at a small scale laboratory system

This project focussed on two organs; corpus luteum and liver. We demonstrated the ability to disaggregate luteal cells from sheep corpus luteum using an established protocol. We developed processes for the isolation of luteal cells from mixed cell types and showed the ability to culture these cells *in vitro* for more than one week in culture. An enzyme-linked Immunosorbent assay (ELISA) was developed to quantify the progesterone levels in the cell culture medium from control and stimulated cells. Upon stimulation of the luteal cells in culture with a single stimulus (luteinizing hormone, 22-Hydroxy cholesterol or dibutyryl-cyclic adenosine monophosphate), progesterone levels were amplified more than 10-fold above baseline levels. The combination of two stimuli produced a synergistic effect, whereby progesterone levels were amplified on average above 40-fold and up to 90-fold for individual luteal cell preparations. We were also able to scale the process up from a small laboratory scale tissue culture plate (surface area of 0.09cm^2) to a 1950-fold increase in surface area (175cm^2) and demonstrate the ability to culture the cells and amplify the production of progesterone under these conditions.

For this project, we needed to develop a high-throughput disaggregation method for the isolation of liver cells. We developed a novel method that efficiently and rapidly disaggregated liver tissue and yielded high numbers of viable cells, consistent with the established perfusion method. The new method needed to preferentially collect the bioactive-producing hepatocyte cells from the liver so we developed a slow centrifugation step which generated near pure hepatocyte samples. These cells maintained cell viability when cultured *in vitro* for more than one week in culture in serum-free

medium. The use of serum-free medium will result in more efficient and cost-effective methods for the commercial purification of target bioactives. The detection and quantification of sheep liver bioactives required the development of analytical methods. We developed ELISA assays for fibroblast growth factor-2 (FGF2) and platelet-derived growth factor (PDGF) and demonstrated the ability of these assays to detect the sheep isoform of these secreted proteins. The amplification of FGF2 and PDGF was demonstrated in sheep liver cells of 20.3-fold and 4.9-fold using serum and retinoic acid, respectively. Furthermore, we demonstrated the ability to amplify both FGF2 and PDGF using the same stimulus indicating the ability to amplify more than one bioactive target simultaneously. Liver cells were able to be cultured in both small laboratory scale plates and large tissue culture flasks (a 1950-fold increase in surface area), demonstrating the scalability of the process. Finally, we developed an integrated process for the production of bioactives from liver tissue, demonstrating with four independent liver samples that the organ collection, tissue disaggregation, cell culture and amplification of FGF2 and PDGF were feasible with two independent stimuli.

The ability to disaggregate organs for the collection of functional cells, the successful culturing of the cells *in vitro* and amplification of target bioactives demonstrates the proof-of-concept. The next stage of this project is to further this research and develop it with a commercially viable target bioactive and fast-track the transfer of this technology to industry. The ability to add value to the meat industry by product diversification into the bioactive market will provide great opportunity for the meat processor and the bioactive market to access this alternative technology for the production of valuable bioactives.

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1 Background

1.1 Introduction

1.1.1 Organ Culture

The overall aim of this project is to amplify the yield of commercially valuable bioactive products from farmed animal organs. This project aims to demonstrate a proof-of-concept that it is feasible to use animal organs for the efficient and economic amplification of high value bioactive compounds.

Organ culture often refers to the cultivation of whole organs *in vitro* however it is not likely to be a viable choice for the efficient production of bioactives. The primary reason is that solid organs of large size prevent efficient gaseous diffusion and the exchange of nutrients and metabolites, therefore the capacity for cell survival and synthesis of bioactives will be largely discounted.

Animal organs produced as by-products of the meat processing industry are important sources of valuable bioactives, with potential uses in the pharmaceutical, cosmetic and nutraceutical industries. The spectrum of these bioactives includes proteins, glycoproteins, polypeptides, growth factors, lipids, carbohydrates, and secondary metabolites. While the organs harvested after slaughter could be directly used for bioactives extraction, great potential exists to add significant value by increasing production yield of bioactives using *in vitro* culture.

The cells from animal organs obtained after slaughter can be viable and metabolically active for several days. Considerable effort was devoted to establishing *in vitro* whole organ culture before immortalised mammalian cell lines became widely available in early 1980's. A wide range of organs from bovine, ovine, mouse, rat and human sources have been successfully cultured *in vitro* (Guinazu, Richter et al. 2002; Placzek, Jessel et al. 1993; Schobitz, Gonzalez et al. 2001; Lee, Latridis et al. 2006; Gahwiler 1989; Saitoh, Yasaka et al. 1999; Moseley, Waddington wt al. 2003; Kosazuma, Hashimoto et al. 2004; Kolesnichenko & Popova 1976; Colon, Bhol et al. 2001; Tavakkol, Varani et al. 1999). However, the majority of these organ cultures were used at the laboratory scale to study organ responses to hormones and chemicals in medical or toxicological applications. Therefore, the commercial potential of using *in vitro* culture of cells from harvested organs remains to be demonstrated for the production of mammalian tissue-derived bioactives.

There is evidence in the scientific literature demonstrating that bioactive compounds can be synthesized *in vitro*. For instance, the isolation and *in vitro* culture of bovine luteal cells has demonstrated an increase in progesterone production of 2 to 50 fold in culture (Young, Luderer et al. 1995; Young, Menadue et al. 2005; Rodgers, Lavranos et al. 1995; Rodgers, Vella et al. 1995). Though this success is limited to a research exercise, it provides strong evidence that the amplification of bioactive yield is feasible.

An alternative method for the production of bioactives is to use immortalised mammalian cell lines in bioreactors. The current best immortalised mammalian cell culture systems begin the culture with 1×10^5 cells/ml with an increase in cell density to 1×10^7 cells/ml over 2-3 weeks. However, the volume of 1×10^7 cells/ml cells in a bioreactor corresponds to only 2-3% of the total culture volume, and the capital costs to achieve this high-density are extremely high (Wurm 2004). It would be far more cost-effective to start the bioreactor process with a high density of isolated cells from harvested organs.

Millions of tonnes of mammalian organs are produced as low-value by-products of the meat processing industry annually. For instance, Australia produces ca. 27,400 tonnes liver from beef and veal each year at a market price of less than $2/kg. Therefore, the cost of these metabolically active cells from harvested organs is only a tiny fraction of immortalised mammalian cell culture. As these organs are available year round, significant economic advantage can be expected to accrue to the meat processing industry if high-value bioactive products can be produced by culturing organ-derived cells.

1.1.2 Using Organs for Bioactive Production

Cells that make commercially valuable bioactives, are specialised, differentiated, and tend not to divide or proliferate. The lack of proliferation is not a problem in this project, because there is a constant supply of cells from the meat processing industry. The *in vivo* cellular production of bioactives with commercial potential is largely dependent upon the three dimensional environment that the cells normally live in. For example, liver hepatocyte cells make the commercially valuable bioactive compound, transferrin (Goldsworthy, McCartor et al. 1970). In the liver they are in contact with other hepatocytes as well as endothelial cells that form the capillaries and other blood vessels, and with migrant cells of the immune system that secrete growth factors and cytokines. Hepatocytes (and specialised cells from other tissues) are embedded within an extracellular matrix that is a three dimensional scaffold made up of long chain molecules that include collagen and fibrinogen. They are also bathed in a dynamic solution derived from lymph and blood, which contains various signalling and regulatory molecules. When cells are isolated out of this three dimensional architecture and cultured *in vitro,* their ability to make the bioactives that they made *in vivo* is often reduced and is usually dependent upon culture conditions that facilitate their attachment, or adherence, to a replacement scaffold - usually the surfaces, floors and walls, of the cell culture vessel. Cell adherence in research-based cell culture is often promoted by coating the cell culture vessel with collagen or fibrinogen.

1.1.3 Optimisation of Cell Culture Conditions

Animal organs or tissues do not consist of one cell type, but are a mixture of cell types. Most tissues contain a population of specialised cells, for example luteal cells in the corpus luteum or hepatocytes in the liver. Tissues and organs also contain unspecialised cells that are usually highly proliferative, for example endothelial cells and fibroblasts. Disaggregating a tissue or organ therefore results in a mixed population of cells. When these are cultured *in vitro*, the unspecialised cells that can divide and proliferate increase in numbers and overgrow and out-compete the specialised non-proliferative cells. Furthermore, these organs are constantly supplied with oxygen and nutrients from red blood cells. It has been established that the presence of red blood cells in cell culture can be toxic to the cells as the red blood cells die. Various isolation procedures can be used to separate the specialised from other cells present as a result of disaggregation of the organ. It is not known if it will be necessary to resort to these lengthier isolation procedures for production of cells for the bioreactor. It may be possible to accommodate increasing populations of non-specialised cells without compromising specialised cell-bioactive production. It is also possible that retention of the non-specialised cells will promote specialised cell function and production.

1.1.4 Amplification of Bioactives

One of the main aspects of this project involves identifying methods that promote the amplification or stimulation of bioactives. In the corpus luteum of the ovary, luteal cells make the steroid hormone progesterone. In a fertile cow, the brain-derived luteinizing hormone (LH) stimulates the luteal cells to amplify production of progesterone more than two-fold. In a pregnant cow, embryo-derived chorionic gonadotrophin (CG) stimulates luteal cells to secrete 10 to 20 times more progesterone. These amplification mechanisms are employed routinely in *in vitro* research-based cell culture systems (Young, Luderer et al. 1995; Young, Menadue et al. 2005; Rodgers, Lavranos et al. 1995; Rodgers, Vella et al. 1995). In addition, the starter substrate molecule from which progesterone is synthesised can be obtained cheaply from Sigma and provided to luteal cells in culture systems. This has been demonstrated to increase progesterone production 2 to 50 fold. The luteal cell response to LH or CG is mediated by the same LH/CG receptor, but cell isolation techniques that free the luteal cells from the extracellular matrix often compromise or strip off those receptors, and so reduce the effect of progesterone amplification signals. The ability of these cells to utilise the starter substrate molecule, however is not mediated by a receptor. In summary, production of bioactives by specialised cell types is usually stimulated by the activation of cell surface receptors. So those receptors must be maintained during the preparation and culture of the cells.

2 Objectives

The overall aim of this project is to amplify bioactive products from animal organs as a proof-of-concept. We aim to develop protocols for the amplification of at least one bioactive target (preferably two) from two different organs. We opted to demonstrate the proof-of-concept from the corpus luteum and develop methods for the disaggregation and amplification of bioactives from liver tissue for a model for commercialisation. The specific aims of this project included:

- Compile a comprehensive literature and patent review discussing the sources of synthesis of bioactives in organs, primary cell culture of corpus luteum and liver, the processes of stimulating and analysing target bioactivity and the available bioreactor systems that could be used for liver cell culture in a large-scale format.
- Establish methods for the collection and transport of organs from the meat processing facility to the laboratory
- To develop and optimise the organ disaggregation protocol that attained high yields of viable cells
- To develop a plate cell culture format that allows for the efficient isolation of luteal or liver cells and promotes the efficient attachment of the cells to a small scale cell culture flask
- To develop various analytical techniques that allow for the detection and quantification of the target bioactives
- To identify and demonstrate the amplification of bioactive production in a small-scale laboratory format
- To optimise the attachment of cells to tissue culture dishes and flasks by assessing different culture matrices that promote adherence
- To develop disaggregation methods for a scalable process with liver tissue
- To demonstrate the amplification of target bioactives in a large-scale format

- To demonstrate the development of an integrated process from organ collection to bioactive amplification in a large-scale format with liver tissue

The demonstration of these specific aims will demonstrate the proof-of-concept of the feasibility of amplifying bioactives from animal organs to add value to the meat processing industry.

3 Methodology

The main stages of the process are illustrated below:

The detailed protocols and developed Research Operating Procedures for each stage of the process are presented in Appendix 1 of this report.

3.1 Corpus Luteum

3.1.1 Organ Collection

The collection of ovaries (corpus luteum) was performed at the meat processing facility, T&R following guidelines and Occupation Health and Safety Policies as per Research Operating Procedure 1: T&R Protocols. The collection of corpus luteum required the sterilisation of equipment, such as scissors, scalpel blades, forceps and collection pots. Sterile transport medium (HBSS) was prepared and 10ml was chilled to 4^{O}C overnight in the collection pots. The reproductive system was collected in sterile trays and the ovaries removed using scissors and immediately placed into the collection medium, which was stored on ice. The unused portion of the organ was returned to the line and the organs transported to the laboratory on ice as detailed in Research Operating Procedure 2: Collection of Animal Tissue.

3.1.2 Disaggregation of Corpus Luteum

The collected corpus lutea were transferred to a sterile biohazard cabinet and all manipulations performed from this point forward were completed under these conditions. The CL was sliced and the halved corporal luteum was shelled into a sterile petri dish. The CL was sliced as thinly as possible using scalpel blades and the exact weight of CL tissue recorded. The CL was disaggregated using 10ml of 400U/ml of collagenase per gram of tissue. Disaggregation occurred at 37^{O}C with gentle shaking for a total time of 2 hours and 40 minutes, with fresh collagenase added after 40 mins. Cells were collected using a plastic transfer pipette and transferred to a 10ml centrifuge tube. Cells were collected by centrifugation at 200xg for 10 mins. Cells were resuspended in DMEM containing 10% FCS as detailed in Research Operating Procedure 3: Disaggregation of Corpus Luteum.

3.1.3 Isolation and Plating of Luteal Cells

Luteal cells were isolated from other cell types, such as red blood cells by layering the cells on top of a ficoll density gradient as detailed in Research Operating Procedure 4: Isolation of luteal cells.

The resultant cell suspension was washed several times and cells resuspended in DMEM containing 10% FCS. Cell numbers and cell viability was assessed by counting cells under a haemocytometer using the trypan blue exclusion test as per Research Operating Procedure 5: Cell and viability counts using a haemocytometer.

Isolated luteal cells were plated into 96 well plates, 6 well plates or T25, T75 or T175 flasks at approximately 6.67 x 10^4 cells per cm^2 as per Research Operating Procedure 6: Plating of luteal cells into cell culture flasks.

3.1.4 Amplification of Progesterone from Sheep Luteal Cells

Stimulatory factors were dissolved in the correct solvent and master mix solutions of the correct medium containing the stimulatory factor were prepared following Research Operating Procedure 7: Stimulation of progesterone from sheep luteal cells. The fresh medium containing the stimulatory factor was added to the flask and cells incubated for 48 hours at 37^oC, 5% CO_2. The medium was collected and assayed by RIA or ELISA.

3.2 Liver

3.2.1 Organ Collection

The collection of liver was performed at the meat processing facility, T&R following guidelines and Occupation Health and Safety Policies as per Research Operating Procedure 1: T&R Protocols. The collection of liver required the sterilisation of equipment, such as scissors, scalpel blades, forceps and collection pots. Sterile transport medium (RPMI) was prepared and 10-150ml was chilled to 4^oC overnight in the collection pots, depending on the amount of liver required. The liver was collected in sterile trays and liver segments removed using scissors and immediately placed into the collection medium, which was stored on ice. The unused organ was returned to the line and the organ transported to the laboratory on ice as detailed in Research Operating Procedure 2: Collection of Animal Tissue.

3.2.2 Disaggregation of Liver Tissue

Liver tissue was disaggregated using both small and large scale disaggregation methods, which is outlined in Research Operating Procedure 11: Mincing of liver samples and Research Operating Procedure 12: Disaggregation of liver tissue. Small scale disaggregation involved using a small segment of liver (1gram) and slicing it as thinly as possible with a scalpel blade and then dicing it to give liver tissue pieces of sizes of approximately 1mm^3. Liver tissue was transferred to a 50ml conical flask and the exact weight of the liver recorded. RPMI was added to the liver for storage overnight at 4^oC. Large scale disaggregation involved using a mincing machine that minced the liver tissue into pieces of approximately 3-5mm^3. 30-40 grams of tissue was collected and transferred to a 200ml conical flask, with the exact weight of liver recorded. RPMI was added to the liver for storage overnight at 4^oC.

The liver tissue was disaggregated using a 0.05% collagenase solution, with 4 separate collections of 30 minutes duration each (total of 2 hours disaggregation). The disaggregated liver cells were

collected by transfer pipette (small scale) or after passing the liver tissue through a metal strainer and collecting the cells in a sterile beaker (large scale). The collected cells were pelleted by centrifugation and washed in HMM containing 20% FCS.

3.2.3 Isolation and Plating of Liver Cells

Liver cells were isolated from contaminating red blood cells by using two centrifugations at 50xg and resuspending the cells in serum-free HMM. Any remaining liver tissue was removed by passing the cell suspension through a cell filter mesh as detailed in Research Operating Procedure 13: Isolation of liver cells. Cells numbers and viability were assessed by Trypan blue exclusion assay using haemocytometer as per Research Operating Procedure 5: Cell and viability counts using a haemocytometer.

Liver cells were plated into 96 well or 6 well plates or T25, T75 or T175 flasks at a cell density of approximately 2.1×10^5 cells per cm^2 as per Research Operating Procedure 14: Liver cell plating.

3.2.4 Amplification of Target Bioactives from Sheep Liver Cells

Stimulatory factors were dissolved in the correct solvent and master mix solutions of the correct medium containing the stimulatory factor at the correct concentration were prepared following Research Operating Procedure 15: Stimulation of liver cells. The fresh medium containing the stimulatory factor was added to the flask and cells were incubated for 48 hours at 37^oC, 5% CO_2. The medium was collected and assayed by ELISA.

3.3 Analytical Methods

3.3.1 ELISA

ELISA (Enzyme-linked Immunosorbent Assay) was the optimal method for the detection and quantification of target bioactives as it is a highly specific method that allows for high through-put and accurate quantification, particularly at low concentrations. ELISAs for the detection of progesterone, fibroblast growth factor-2 and platelet derived growth factor were developed in the lab as no commercial kits were available to detect the sheep isoforms.

The progesterone ELISA is a competition ELISA, whereby a capture antibody is coated into 96 well ELISA plates and the standards / samples are added with a second progesterone antibody that competes for the same site as the capture antibody. The protocol is performed as per Research Operating Procedure 8: Analysis of progesterone by ELISA and unknown sample concentrations are measured from a standard curve.

The FGF2 ELISA utilises the R&D FGF2 ELISA kit using a bovine standard to generate a standard curve. The ELISA is a sandwich ELISA which involves the use of two antibodies that detect different regions of the same protein, one antibody captures the protein, the other allows for detection using a chemical reaction, as described in Research Operating Procedure 16: basic fibroblast growth factor ELISA.

The PDGF ELISA functions similarly to the FGF2 ELISA and utilises a PDGF ELISA kit purchased from R&D Systems. However, the kit does not detect the sheep isoform and an antibody purchased

from Abcam (ab38561; a polyclonal antibody to PDGF-BB – Carboxyterminal end) was substituted as the capture antibody. This adapted ELISA can detect the sheep isoform of the protein and the protocol is detailed in Research Operating Procedure 17: Platelet Derived Growth Factor ELISA.

3.3.2 RT-PCR

Alternate detection methods were sought for the detection of target bioactives. The use of PCR technology to detect the expression of the gene was established for the detection of FGF2, PDGF, IGF2, TNF-α and transferrin by isolating RNA from cells and performing reverse transcription and gene specific PCR and measuring gene expression levels by agarose gel electrophoresis and ethidium bromide staining. However, due to the establishment of the FGF2 and PDGF ELISA, and the inability of RT-PCR to provide accurate quantification this method was not further utilised.

3.3.3 Radio Immuno Assay

The detection of progesterone levels in cell culture medium was initially assed by an established RIA method in the Department. However, due to the inconsistent quality of an essential component of the assay from the supplier, the established ELISA method became the method of choice.

3.3.4 Identification by 2-D Gel Electrophoresis

The use of proteomic methods for the detection, quantification and identification of proteins is a method we utilised for the detection of unknown, or non-target bioactives. In this process, medium from a control liver sample and a stimulated sample were collected and the protein precipitated. Due to the large amount of albumin present in the medium, we removed the albumin by a number of methods, including precipitation with ethanol and column purification, however the samples still had levels of albumin that were too high for analysis by 2-D gel electrophoresis. Due to the lack of time and loss of resources, this exciting phase of the project had to be put on hold. This aspect of the project will be further assessed into the next phase of the project to attempt to identify novel bioactive compounds of interest.

3.3.5 Chemical Methods

We also attempted to develop methods for the extraction, identification and quantification of bioactives using chemical methods. We developed methods for the detection of several steroidal hormones from luteal cells (oestrone, progesterone, pregnenolone and 17-α-hydroxypregnenolone) simultaneously using HPLC. We were able to develop methods for the extraction (using Solid Phase Extraction) and detection of these compounds.

For the detection of the target bioactives from liver, we attempted to set up a similar detection method that was established for the luteal cell bioactives. We were able to detect the recombinant standards of IGF2, PDGF and FGF2 by MALDI though no targets were detectable by SALDI. We were unable to detect the secreted protein in the medium by HPLC due to the low concentration present and the inability to detect the levels by MALDI or LC-MS due to the poor signal to noise ration. Therefore the detection and quantification of liver bioactives was achieved by ELISA.

4 Results and Discussion

4.1 General Achievements

4.1.1 Literature Review

One of the first major achievements of this project was the Literature Review. This review referenced over 550 scientific journal publications and 20 patents from 1963 to current day. The literature review detailed the locations of where bioactives are synthesised for 24 different organs and we opted the corpus luteum and liver as proof-of-concept target organs. The corpus luteum was chosen due to the expertise available within the Department, the easy identification of the organ and the large number of bioactive compounds synthesised (27). The liver was chosen as the second organ due to its large biomass, the large number of identified bioactives that it synthesises (130) and the available expertise within the University. We identified 25 potential target bioactives that were synthesised in the liver and/or corpus luteum.

We identified a disaggregation method for liver tissue using perfusion of the liver but this method would not be suitable for a scalable process and commercialisation. The requirement for a novel disaggregation method for the isolation of liver cells would be required. Notably, using the perfusion disaggregation method yielded 2.1×10^7 to 3.9×10^7 cells per gram of tissue, with cell viabilities of 82% to 88% (Bakala, Karlik et al. 2003). The literature survey also revealed that liver cells are cultured in media that range in composition from a very basic to very complex and this was often dependent upon the goals of the research articles. The culturing of cells was performed on a number of substrates, including tissue culture plastic and collagen (Hou, Kunitake et al. 2001; Schmid, Reinecke et al. 2000; Du, Chia et al. 2006; Tuschl and Mueller 2006; Mizuguchi, Hui et al. 2001; Smith and Mooney 2007; Pie, Baek et al. 2002; Pasco, Boyum et al. 1988; Nahmias, Kramvis et al. 2006; Weiss, Jahn et al. 2002; Kono, Yang et al. 1997; Blanchard, Alexandre et al. 2005; Selden, Chalmers et al. 2003) and these cells could be cultured up to 123 days (Selden, Chalmers et al. 2003). Several articles also demonstrated differences in functionality between ages of animals and differences between species (Rose, Kostrubsky et al. 2006), indicating that comparing functional results between species may be difficult.

The detection and amplification of a number of target bioactives was researched and discussed in the Literature Review. The ability to develop analytical methods for the quantification of FGF2 and PDGF resulted in these bioactives forming the focus of this project. FGF2 is synthesised in the liver and is one of the major sites of production (Peng, Wang et al. 2005). Amplification of FGF2 has been demonstrated in many cell types by multiple activators. Retinoic acid has been shown to induce FGF2 expression by 10-fold in animal cell lines (Gaetano, Catalano et al. 2001). PMA and arachidonic acid have been shown to induce FGF2 mRNA expression by 9-fold and 2-fold, respectively (Moffett, Kratz et al. 1998; Zeng, Yellaturu et al. 2002). Low oxygen tension has also been demonstrated to increase FGF2 expression by more than 4-fold at the mRNA level within four hours of exposure (Sakaki, Yamada et al. 1995; Le and Corry 1999).

PDGF has been detected in human liver samples and has been secreted in the culture medium of human liver cells (Marra, Choudhury et al. 1994). PDGF has been shown to be induced by serum at the mRNA level (Takimoto and Kuramoto 1995) and glucose induced expression more than two-fold

(Mizutani, Okuda et al. 1992). The addition of dexamethasone was shown to induce mRNA levels by 20-fold above baseline levels (Haynes and Shaw 1992), indicating the possibility of amplifying PDGF expression from liver cell culture.

4.1.2 Collaborations and Organ Transport

A formal approach was made to T&R Pastoral Pty Ltd, Murray Bridge through their Operations Manager to establish their collaboration with this project and allow the research team regular access to the raw materials. Prior to access to the processing floor, all collection staff were required to be vaccinated against Q-fever and to undergo induction under the Occupational Health and Safety guidelines of T&R Murray Bridge.

We established collection methods that used a sterile medium (HBSS or RPMI). This medium facilitated a high cell viability through an even temperature distribution, the prevention of cell dehydration and the maintenance of physiological conditions, such as osmolarity and pH. The aim of collection and transport was to cool the cells quickly to reduce metabolic activity. The inclusion of serum or growth factors was not required. Cells that were disaggregated from tissue collected and transported in this way were observed to maintain viability and remain sterile (Milestone 1.2).

4.1.3 Commercialisation Support

Throughout this project we have provided MLA with commercialisation support for the continuation of this project as a new value adding process for the meat processing industry. We have presented our research at the annual MLA bioactives workshop and engaged with industry at this networking event. We prepared documents and presentations for industrial engagement with Flinders and this research project, and have initiated industry commitment to the technology through a meeting with MLA, T&R and Ralph Meat Co. We have also engaged with MLA and a patent attorney for the application of a provisional patent.

4.1.4 Research Operating Procedures

During this project we have standardised many protocols for the collection, isolation and culturing of liver and luteal cells. When standardising collection protocols we have considered the need to integrate future commercial activities with existing meat processing procedures. We have also developed methods for the detection and quantification of target bioactives. Each of these protocols has been established into a Research Operating Procedure and we have developed 17 protocols throughout this project. These protocols are shown in Appendix 1 of this report.

4.2 Corpus Luteum

4.2.1 Disaggregation of the Corpus Luteum

The disaggregation of the corpus luteum is a well established protocol within the Department. Disaggregation is accomplished by the dissection and slicing of the tissue as thinly as possible and incubating in 10ml of 400U/ml collagenase per 1g of tissue. The tissue was disaggregated for 2 hours and 40 minutes at 37^{O}C with shaking following the addition of fresh collagenase solution after

40 minutes as per Research Operating Procedure 3: Disaggregation of Corpus Luteum. The resultant cell population were collected by centrifugation and cell counts performed using trypan blue exclusion assay following Research Operating Procedure 5: Cell and Viability counts using a haemocytometer.

The corpus luteum samples produced single cells or small clumps of cells (2-6 cells) with high viability. Qualitative assessment of the luteal cells under the haemocytometer revealed that both steroidogenic cell types (large and small luteal cells) were present. Generally, 5 to 8 x 10^6 cells were isolated from each gram of disaggregated tissue with viability higher than 85% (Milestone 1.3). Typically, more than 50% of the liver tissue was disaggregated as measured by the weight of the tissue (Milestone 1.5).

4.2.2 Isolation, Culture and Survival of Luteal Cells

The disaggregation of the corpus luteum resulted in a population of steroidogenic cell types that also contained a large number of red blood cells. To determine whether the presence of red blood cells in the cell culture affected the viability of the luteal cells, red blood cells were removed by using a ficoll density gradient (Research Operating Procedure 4: Isolation of luteal cells) and plated into 96 well plates following Research Operating Procedure 6: Plating of luteal cells into cell culture flasks. Cells were assayed by MTT assay (Research Operating Procedure 10: MTT assay) and it was observed that cell survival was independent of whether the red blood cells were removed or not 24 or 72 hours post-seeding (Milestone 1.4). This data demonstrates the ability to culture sheep luteal cells for at least three days *in vitro*.

4.2.3 Cryogenic Freezing of Luteal Cells

Because luteal cells are seasonal, we sought to find a method of maintaining a constant supply of cells when the sheep were no longer in breeding season. One method to achieve this was to isolate large numbers of luteal cells and freeze them cryogenically. We assessed freshly collected luteal cells for cell survival and ability to respond to external stimuli for the amplification of progesterone expression. Freshly isolated luteal cells maintained survival for at least five days in culture and demonstrated an increase in progesterone levels of up to 15-fold, 12.9-fold and 4.3-fold with LH, 22-hydroxy cholesterol (22H) and dibutyryl cyclic-AMP (db-cAMP), respectively (Figure 1 and Milestone 4.1).

Luteal cells were frozen in 10% DMSO as described in Research Operating Procedure 9: Freezing and thawing of sheep luteal cells. Thawing of the cells revealed greater than 75% of the cells were recovered at a viability higher than 80%, indicating that the cells remained viable after the freeze-thawing process. When these cells were plated into 96 well plates, the cells remained viable for five days in culture though an increase in cell number was observed at day 5, which may be attributable to the proliferation on non-steroidal cell types (Milestone 1.5).

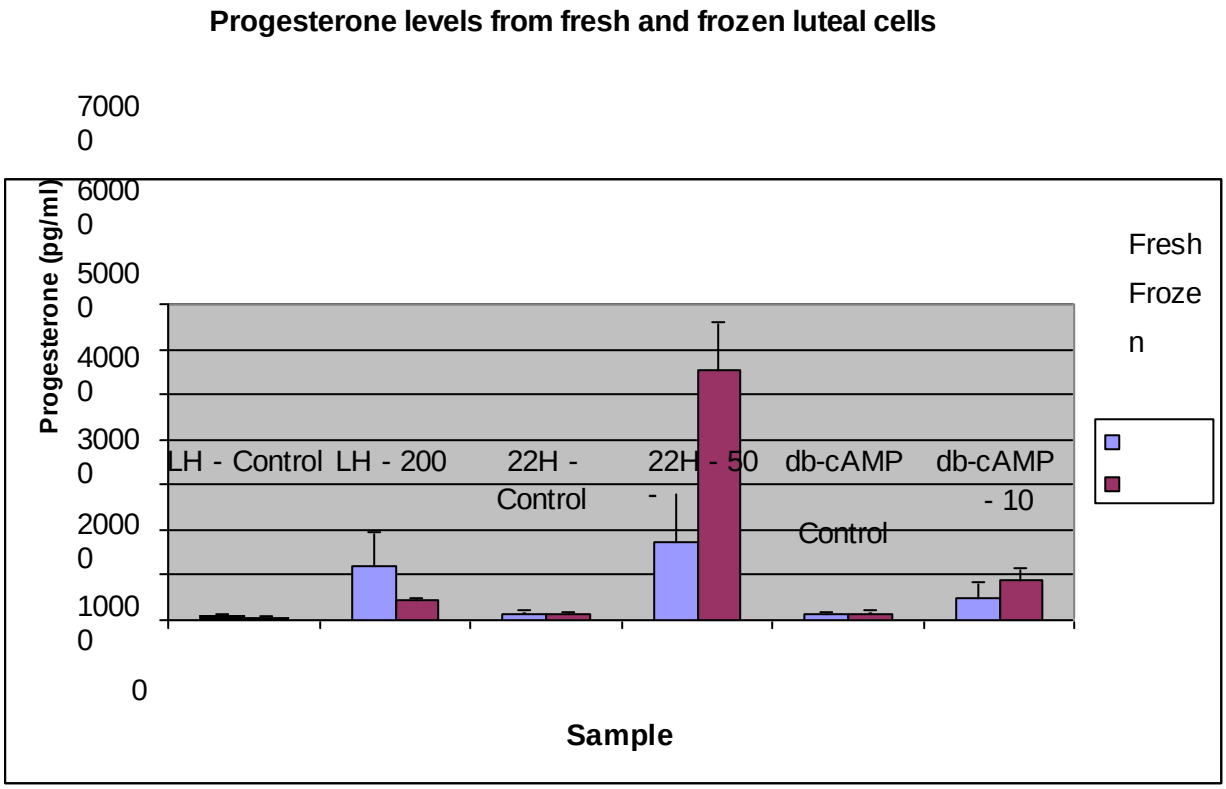


Figure 1. Freshly isolated luteal cells (Blue bars) or frozen and thawed cells (purple bars) were plated into 96 wells plates at 20,000 cells per well. Medium containing a stimulus (LH at 200ng/ml; 22H at 50µM; db-cAMP at 10µM) was added 24 hours later and medium collected and subject to Progesterone ELISA 24 hours later. Data is represented as the mean ± standard deviation from 3 independent wells.

Figure 1 shows that luteal cells can be induced to increase the production and secretion of progesterone into the culture medium as measured by ELISA with any of the simulants tested. The baseline levels were approximately 1000pg/ml and the addition of LH to the culture medium resulted in an increase in the level of progesterone detected by up to 15.1-fold, yielding levels of 12ng/ml. Stimulation of luteal cells with 22H resulted in a maximal stimulation of 12.9-fold with 50µM, yielding 17ng/ml. Stimulation with db-cAMP was less efficient at inducing progesterone, with a modest increase of 4.3-fold at the highest concentration tested. The addition of stimuli to frozen and thawed cells resulted in an induction of progesterone which was overall relatively similar when compared to the fresh samples. Some stimuli did not induce expression as highly in the frozen cell population, such as LH and 22H which increased progesterone production by 36.4-fold.

Overall, the responses appear to be relatively similar between fresh and frozen and thawed cells, indicating that freezing luteal cells may be a viable option for using these cells all year-round.

4.2.4 Amplification of Progesterone from Sheep Luteal Cells

Functional activity of sheep luteal cells was measured by adding progesterone stimulatory factors to the cell culture medium, including LH, 22H and db-cAMP. The addition of these stimuli had little to no effect on the survival of the luteal cells after 72 hours of culture. The addition of LH to the culture medium resulted in an increase in the production of progesterone in a dose dependent manner (Figure 2).

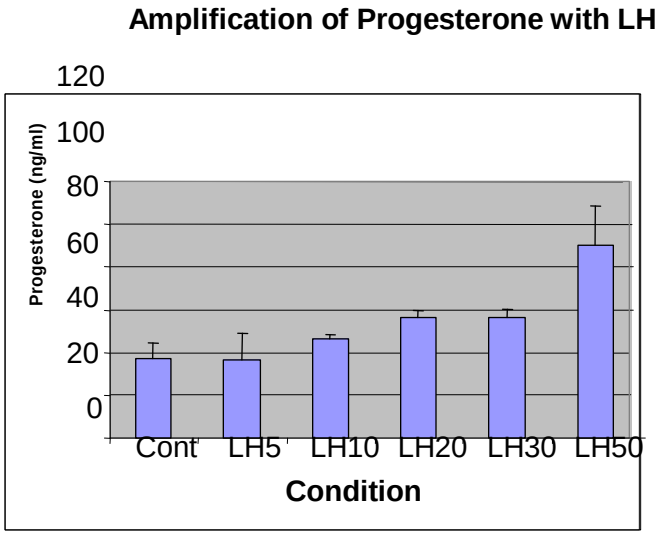


Figure 2. Luteal cells were isolated and red blood cells removed by ficoll density gradient. Cells were grown in the presence of the control (water) or LH (ng/ml). After 24 hours of incubation, the medium was removed and analysed for the amount of progesterone present by RIA. Data is represented as the mean + standard deviation of 3 replicate wells.

Data presented in Figure 2 demonstrates the amplification of progesterone with LH in a dose-dependent manner, with maximal induction at 50ng/ml producing more than 90ng/ml. Surprisingly when luteal cells were stimulated with LH and the red blood cells were not removed, LH had no effect on the production of progesterone, suggesting that the culturing of luteal cells in the presence of red blood cells does not enable them to respond to the agonist (Milestone 1.5).

The next step was to combine the stimulatory factors to determine whether the induction of progesterone could be enhanced further to yield higher amplification of progesterone. A number of combination ranges were tested at various concentrations (Milestone 4.1). Amplification levels of up to 90-fold were observed in several independent luteal cell preparations and Figure 3 demonstrates the average stimulation observed from four independent luteal cell preparations.

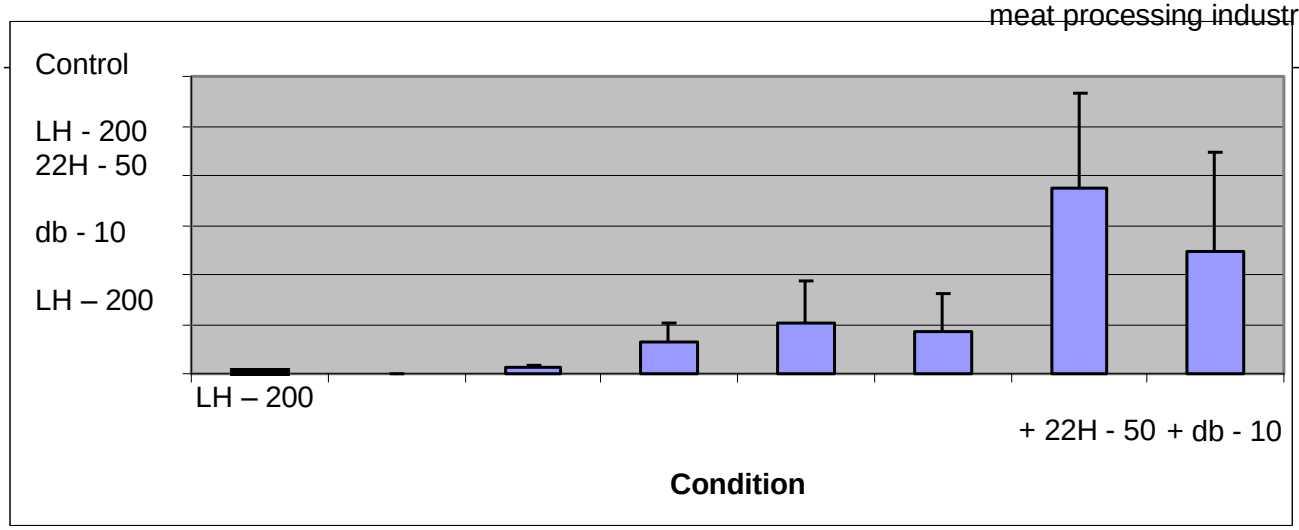


Figure 3. Frozen luteal cells were thawed and plated into 96 wells plates at 20,000 cells per well and medium containing the stimulus at the appropriate concentration was added 24 hours later. The

expected concentration of progesterone extracted from blood and tissue is shown. Medium was collected and subject to Progesterone ELISA 24 hours later. LH was added at ng/ml; 22H was added at µM and db-cAMP was added at µM concentration. Data is represented as the mean ± standard deviation 3 independent wells from 4 independent luteal cell preparations.

The combination of factors should result in an enhancement of activation of progesterone, particularly if different signalling pathways are induced. The level of progesterone in blood is less than 100pg/ml and direct extraction from tissue has not been determined. Luteal cells isolated produce similar levels to that observed in blood. Stimulation with a single stimulus yielded fold inductions above control levels of 7-, 12- and 10-fold for LH, 22H and db-cAMP, respectively. Combining stimuli resulted in a fold induction up to 43-fold, indicating that multiple stimuli can act synergistically for the induction of progesterone.

4.2.5 Detection and Quantification of Luteal Cell Bioactives

At the commencement of this project, a radioimmunoassay was established as a method for the detection and quantification of progesterone. When the progesterone tracer used in the RIA became unreliable, we sought to develop a progesterone ELISA. An ELISA that was developed in Adelaide to detect human progesterone was sourced and we determined that the antibodies could also detect the sheep isoform and was therefore employed as the method of choice for the detection and quantification of progesterone.

An alternative aim of developing analytical methods was to determine an optimal method for the detection of multiple bioactives simultaneously. The first step was to determine the optimal solvent for the extraction of the bioactives from medium for detection by HPLC. Four target bioactives were analysed: Estrone, progesterone, 17-α hydroxyl-pregnenolone and pregnenolone. It was found that using 70% acetonitrile promoted the detection of all four bioactives (Figure 4).

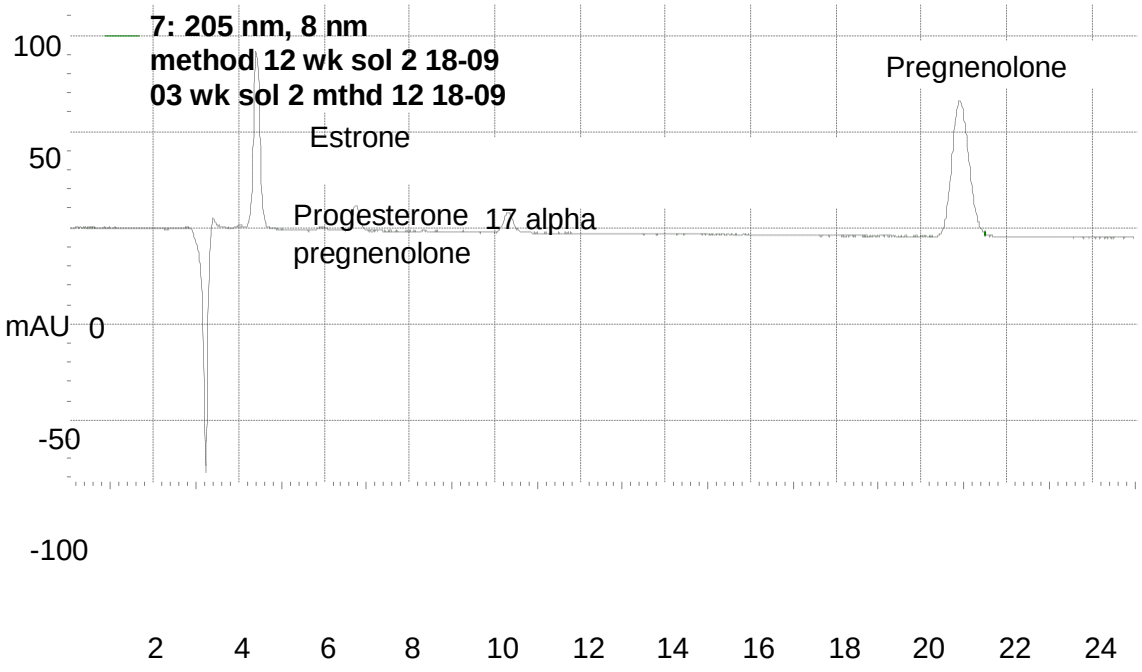


Figure 4. Working solution containing progesterone (5.6µg/ml), estrone (40µg/ml), 17 alpha hydroxy pregnenolone (40µg/ml) and pregnenolone (158.4µg/ml) analysed by elution in 70% acetonitrile.

The preparation of the standard required a pre-treatment using solid phase extraction (SPE). This process was performed to remove any unwanted compounds present in the sample medium before injection into the HPLC. Because there is likely to be some loss of product during SPE, each sample was spiked with the same amount of internal standard and the final concentration of the sample was determined by calculating the target peak to internal standard peak ration. The internal standard chosen was hydrocortisone as it has similar properties to the target bioactives and is produced in the adrenal cortex and therefore will not be present in the samples and will not interfere with the target bioactives.

To determine the limit of detection, a series of standards were prepared by spiking blank medium with the four analytes at concentrations ranging from 1ng/ml to 5000ng/ml. It was found that the lowest concentration that could be detected was 100ng/ml and this is therefore the limit of detection of these bioactives Milestone 1.5). It was found that the recovery of bioactives when using the SPE column was quite high (Table 1).

Standard	**% Recovery**
Progesterone	93
Estrone	74
17-alpha	101
Pregnenolone	88

Table 1. % Recovery from target bioactives after solid phase extraction.

The detection of progesterone was observed in medium from luteal cells stimulated with LH or 22-Hydroxy Cholesterol at both the 24 and 48 hour timepoints, however no detection of progesterone was observed at the 72 hour timepoint. Notably, estrone, pregnenolone nor 17-α hydroxypregnenolone were detected in any sample (Milestone 2). Using this method for quantification resulted in progesterone levels of 2100ng/ml with LH stimulation and 1200ng/ml with 22-Hydroxy cholesterol (Milestone 2).

4.2.6 Scale-up of Luteal Cell Culture

All work presented here and in previous Milestone Reports were all conducted in 96 wells trays, which has a surface area of 0.09cm^2. Luteal cells were cultured in larger flasks and exposed to stimuli to determine whether the amplification of progesterone was possible in larger flasks. Cells were seeded into T25 flasks (277-fold increase in surface area) at a cell density consistent with 96 well trays. Baseline progesterone levels were consistent with that of 96 well trays. Luteal cells were cultured into T175 flasks (1944-fold increase in surface area) and subjected to lower concentrations of stimuli to demonstrate progesterone amplification because of the expense of using a large concentration of LH. 22-hydroxy cholesterol was used at a concentration of 0.2µM and dibutyryl cyclic-AMP at a concentration of 0.1µM and medium harvest daily for 5 days (Figure 5).

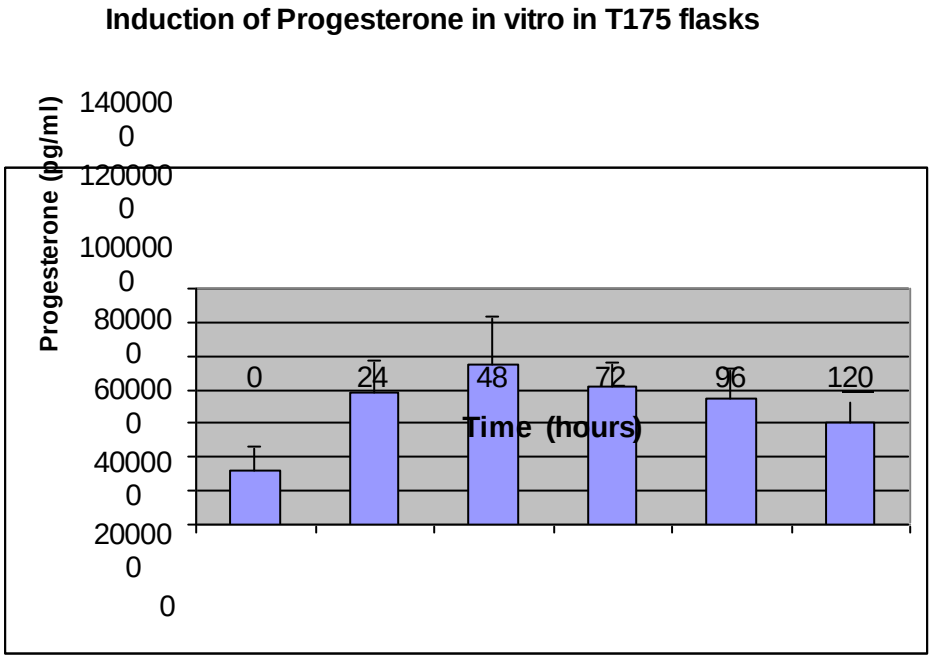


Figure 5. Fresh luteal cells were seeded into T175 flasks at a density of 1.1 x 10^7 cells per flask and incubated overnight. The next day, the medium was collected (time = 0 hours) and fresh medium containing 22H at 0.2µM and db-cAMP at 0.1µM was added to the flask. The next day, medium was collected and the fresh medium containing the stimulatory factors was added. This was repeated for the next 4 days. Medium was subjected to progesterone measurement by ELISA and data is presented as the mean ± standard deviation from duplicate samples of 2 replicate flasks.

The culturing of luteal cells in T175 flasks resulted in the induction of progesterone in the presence of low levels of stimulatory factors, up to 3.1-fold after 48 hours. The level of progesterone slowly decreased over time, consistent with non-stimulated cells in T25 flasks (Milestone 4.1). In this experiment, more than 95ng/ml progesterone was detected, yielding 3.8µg per flask. It can be predicted that the level could be enhanced to more than 1.13mg of progesterone per flask if the optimal stimulatory conditions were used.

This data demonstrates that progesterone can be induced from luteal cells in large scale tissue culture flasks and demonstrates for one organ (corpus luteum) that target bioactives can be amplified from cells isolated from organs collected from the meat processing floor. This observation demonstrates the proof-of-concept.

4.3 Liver

4.3.1 Disaggregation of Liver Tissue

The collection of liver tissue was reported in Milestone 1.2. We found that organs can be collected and transported to the laboratory in cell culture medium (RPMI) on ice and the cells remain viable.

The disaggregation of liver tissue has been reported in the scientific literature to involve perfusion of a disaggregation solution. This process involves using a collagenase solution and pumping it through the liver tissue via the hepatic artery, collecting the solution through the vein and re-circulating the solution through for a period of 20 minutes. This process is not suitable for

disaggregation of liver tissue as required in this project and we therefore sought to find a novel disaggregation method that was scalable and efficient in the isolation of liver cells.

To determine which disaggregation method is optimal, sheep liver tissue was collected and subjected to mincing and different disaggregation solutions for different periods of time. Liver samples were either minced by cutting the tissue into small pieces ($1mm^3$) with scalpel blades or left as an intact piece of liver tissue ($1cm^3$). Liver samples were incubated in different solutions overnight at 4^oC. These solutions included:

- Minced liver sample incubated in RPMI (control)
- Minced liver sample incubated in trypsin-EDTA
- Minced liver sample incubated in collagenase
- Non-minced liver tissue incubated in RPMI (control)
- Non-minced liver tissue incubated in trypsin-EDTA
- Non-minced sample incubated in collagenase

The next day, the liver tissue was disaggregated using trypsin or collagenase (consistent with overnight soaking, with the RPMI controls disaggregated with trypsin-EDTA) and cells were collected every 30 minutes with four collections in total, following Research Operating Procedure 12: Disaggregation of liver tissue. Data presented in Milestone 1.3 showed that collagenase disaggregation was the most efficient in removing cells from the liver portion. Trypsin-EDTA disaggregation yielded fewer cells than collagenase treatment and mincing produced more cells than the non-minced samples (Figure 6). Increasing the surface area for the disaggregation solutions to access the liver tissue is more likely to allow for cells to be more efficiently removed, however this comes at an increased risk of physically damaging the cells and lowering the viability.

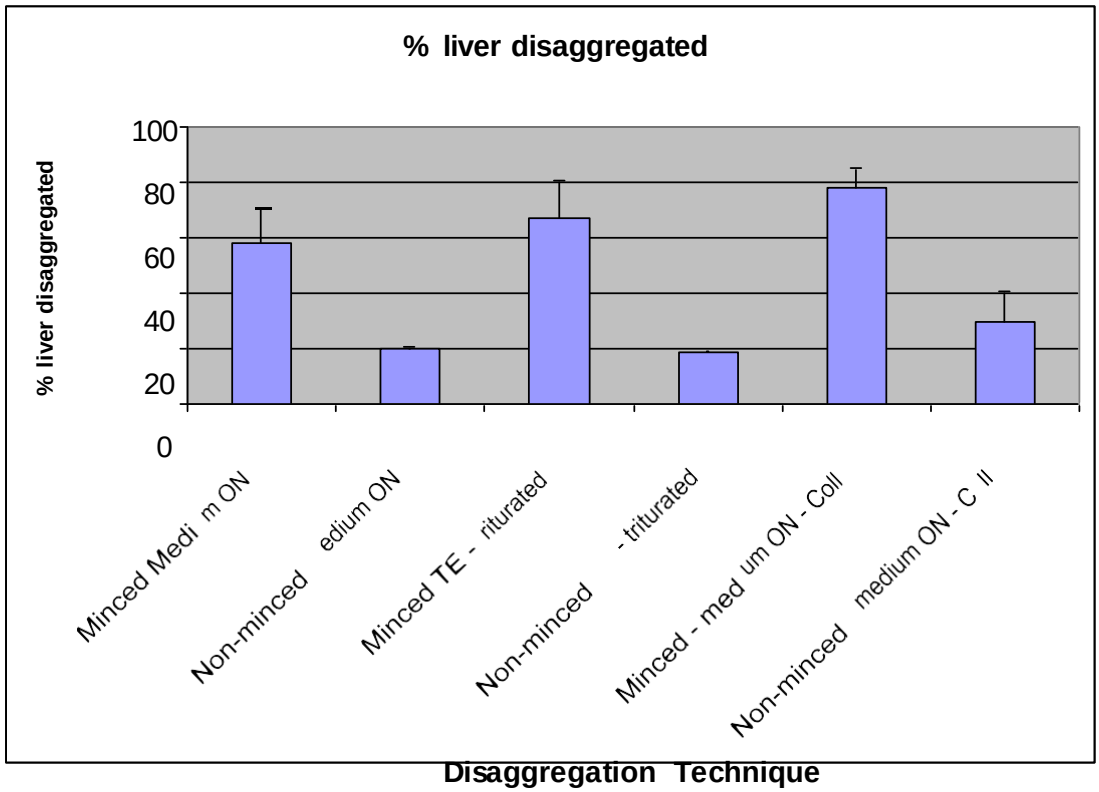


Figure 6. 1g sheep liver samples were minced (samples 1, 3, 5) or non-minced (2, 4, 6) and incubated overnight (O/N) in HMM medium. The next day liver samples were exposed to warm trypsin / EDTA (samples 1, 2, 3, 4) or collagenase (samples 5, 6) and disaggregated for a total time of 2 hours with supernatants collected at 30 minute intervals. Triturated samples were drawn into a transfer pipette several times to dislodge any loose cells from the tissue sample. The weight of the liver post-disaggregation was weighed and the data is presented as the % of liver disaggregated from the initial sample weight. Data is presented as the mean + standard deviation from at least 3 independent experiments.

Figure 6 demonstrates that mincing the samples resulted in a marked increase in the amount of liver disaggregated from the sample regardless of the disaggregation solution. The number of cells collected from the above disaggregation was calculated (Figure 7).

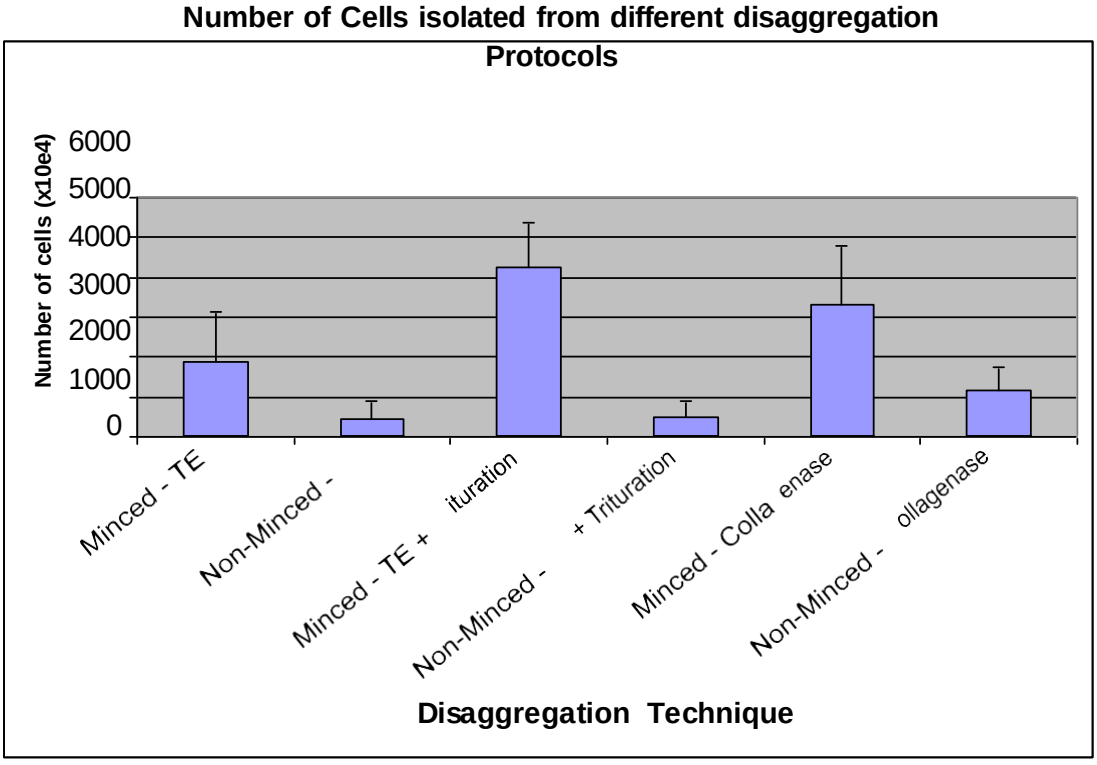


Figure 7. 1g sheep liver samples were minced (samples 1, 3, 5) or non-minced (2, 4, 6) and incubated overnight (O/N) in HMM medium. The next day liver samples were exposed to warm trypsin / EDTA (samples 1, 2, 3, 4) or collagenase (samples 5, 6) and disaggregated for a total time of 2 hours with supernatants collected at 30 minute intervals. Triturated samples were drawn into a transfer pipette several times to dislodge any loose cells from the tissue sample. Total Cell number was counted using a haemocytometer and data is presented as the total number of cells isolated. Data is presented as the mean + standard deviation from at least 3 independent experiments.

Consistent with the liver weight data (Figure 6), the number of cells collected was highest in the minced samples compared to the non-minced samples with each disaggregation solution tested.

The soaking of the samples in disaggregation solution overnight had no effect on the number of cells

collected (Figure 8).

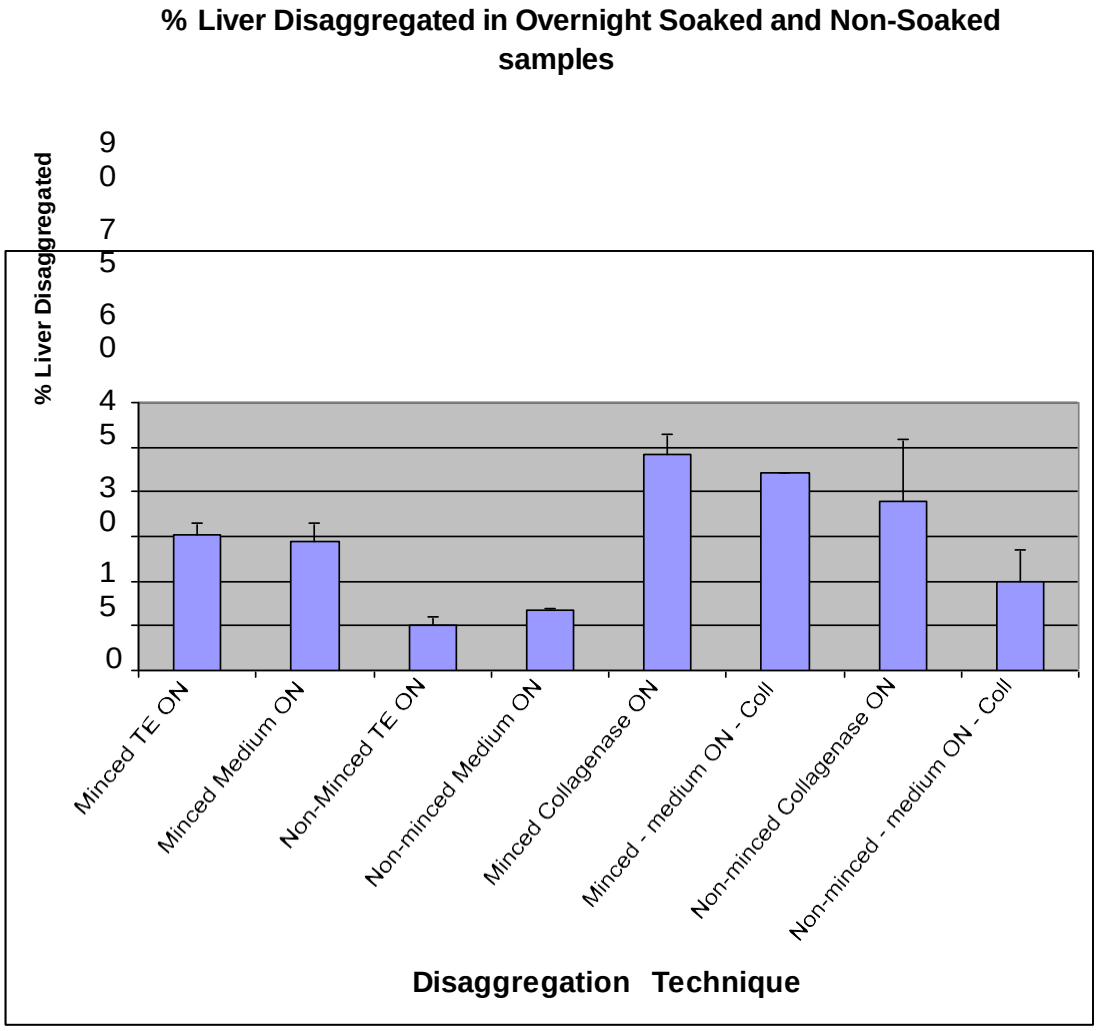


Figure 8. 1g sheep liver samples were minced (samples 1, 2, 5, 6) or non-minced (3, 4, 7, 8) and incubated overnight (O/N) in Trypsin / EDTA (TE) (samples 1, 3), Collagenase (samples 5, 7) or HMM medium (samples 2, 4, 6, 8). The next day liver samples were exposed to warm TE (samples 1, 2, 3, 4) or collagenase (samples 5, 6, 7, 8) and disaggregated for a total time of 2 hours with supernatants collected at 30 minute intervals. The weight of the liver post-disaggregation was weighed and the data is presented as the % of liver disaggregated from the initial sample weight. Data is presented as the mean + standard deviation from at least 3 independent experiments.

Data presented in Figure 8 demonstrated soaking samples overnight in trypsin-EDTA (TE) had little effect on the amount of liver tissue disaggregated when compared to the control liver samples that were soaked in medium. Soaking samples overnight in collagenase resulted in a slight increase in the amount of liver disaggregation when compared to medium soaked samples (compare samples 5 and 7 to 6 and 8). Therefore, the soaking of samples overnight in disaggregation solution had no effect on the amount of tissue disaggregated (Figure 8) or the number of cells collected (Milestone 1.5) and was omitted from the disaggregation protocol.

To determine which disaggregating solution (TE or collagenase) was the most efficient at disaggregating the liver tissue and producing viable cells in a consistent manner, several independent liver samples were tested and data combined to test reproducibility (Figure 9).

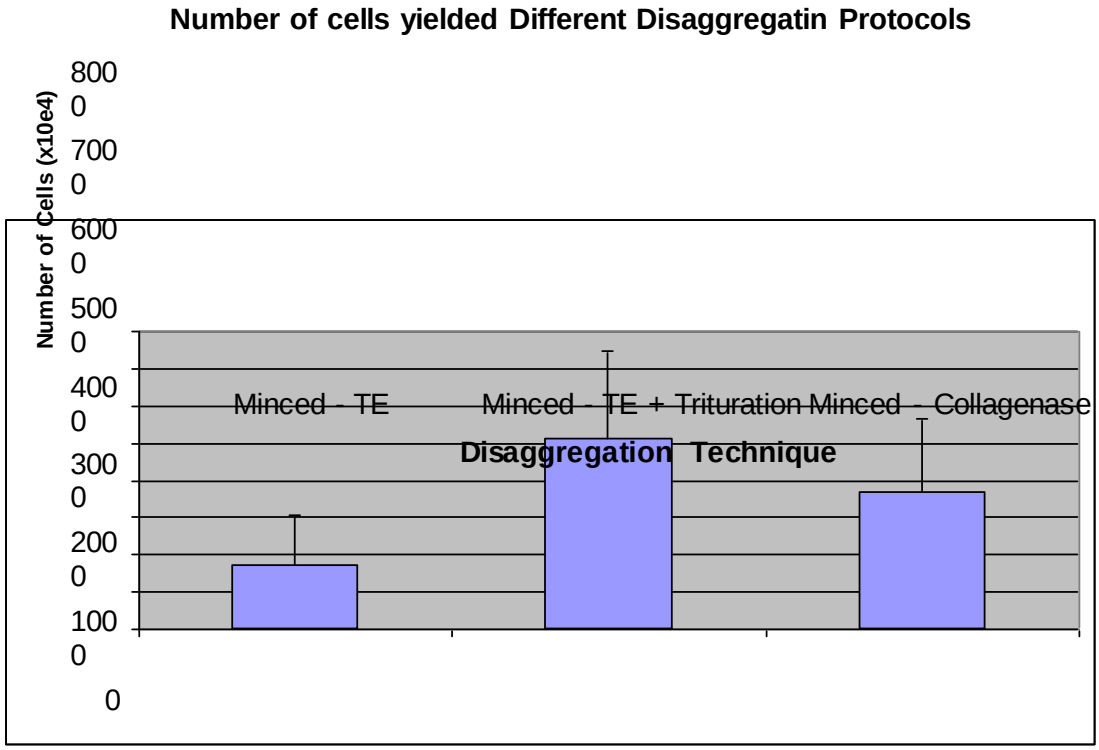


Figure 9. 1g sheep liver samples were minced and incubated overnight (O/N) in HMM medium. The next day liver samples were exposed to warm trypsin / EDTA (samples 1, 2) or collagenase (sample 3) and disaggregated for a total time of 2 hours with supernatants collected at 30 minute intervals. Triturated samples were drawn into a transfer pipette several times to dislodge any loose cells from the tissue sample. Total Cell number was counted using a haemocytometer and data is presented as the total number of cells isolated. Data is presented as the mean + standard deviation from at least 6 independent experiments.

Figure 9 shows that when data from at least 8 independent liver samples is pooled that TE disaggregation with trituration and collagenase disaggregation are more efficient than TE. The total number of cells collected is more variable than the liver weight data (Milestone 1.5) and may be due to post-disaggregation processing. However, qualitative differences were observed between the disaggregation methods with more large cells consistently observed with collagenase disaggregation.

To determine which disaggregation solution tested in Figure 9 was optimal for liver cell recovery, liver cells were disaggregated from five independent sheep liver sample to measure consistency between samples and to also measure whether there are variations between livers from different animals. Livers from five sheep were collected and subjected to disaggregation by TE, TE + trituration or collagenase to assess the consistency of disaggregation. A mix sample of the five livers was prepared by mixing equal portions of the five livers to simulate a mixed liver sample. Each of the three disaggregation methods yielded similar results in regards to the amount of liver disaggregated (Milestone 1.5) indicating the process is robust. The mixed liver sample yielded similar results to that observed when the average of the five independent liver samples was assessed indicating mixing livers will have no effect on the efficiency of liver tissue disaggregation.

Each of the disaggregation solutions yielded similar cell numbers across each of the liver samples and the average of the five livers gave similar results to that of the mixed liver sample. TE disaggregation yielded the lowest number of cells (3.12×10^7 cells) and TE + trituration yielded almost twice as many cells (6.79×10^7 cells) and collagenase yielded the highest number of cells (7.01×10^7 cells) indicating that collagenase disaggregation may be the optimal method.

To assess which method was the optimal method for the disaggregation of liver tissue we sought to measure the number of hepatocytes that were collected by each protocol. Liver tissue was disaggregated using TE, TE + trituration or collagenase and seeded into 96 well plates. Three random microphotographs were taken of cells disaggregated by each method and the hepatocytes in each frame counted. Hepatocytes are readily observable or morphological analysis due to their large size in comparison to other cells present. Hepatocyte cell morphology counts revealed that TE disaggregation with trituration yielded the highest number of hepatocytes however also had the highest variation between liver samples. TE disaggregation produced the lowest number of hepatocytes when compared to the other disaggregation solutions and collagenase resulted in a more consistent hepatocyte yield (Milestone 3).

To identify and measure whether the cells were metabolically functional, an albumin immunohistochemistry analysis was employed. Albumin was used as it is synthesised only by hepatocytes and is a measure of cell functionality. It was observed that each disaggregation method resulted in albumin positive cells. Notably, collagenase disaggregation resulted in the presence of the most number of albumin positive cells (Milestone 3). This data, taken together with the disaggregation and morphology of the cells indicated that collagenase disaggregation was the optimal method for hepatocyte isolation from liver tissue.

4.3.2 Optimisation of Liver Cell Isolation and Cell Culture Format

The removal of red blood cells from liver cells is an important determinant of cell survival as red blood cells can become toxic to other cells if left in the culture for long periods of time. Several approaches for the removal of red blood cells were trialled, including red blood cell lysis, removal by slow centrifugation or ficoll density gradient. Data presented in Milestone 1.4 demonstrated that slow centrifugation and Ficoll density gradient resulted in cell viabilities that were similar to that of untreated cells, whereas red blood cell lysis had a reduced viability count indicating that the lysis solution had some effect on the integrity of the hepatocytes.

The optimal culture of liver cells requires the selection of the correct culture medium, cell substratum and cell density. We examined the effect of different medium on cell survival and viability as well as substrates for the cells to adhere to. We found that cells cultured in 96 well plates with HMM containing 20% FCS showed optimal cell viability and survival and lower cell density had no effect on cell survival.

It was observed that using different substrates for the cells to adhere to had negative effects to cell attachment and survival. Gelatin was found to be a poor substratum, with less than 35% of cells adhering to the well. Notably, collagen coating promoted efficient cell attachment to the culture well however these levels were not higher than that observed for uncoated tissue culture surfaces, indicating that no additional coating was required for the adherence of liver cells to the cell culture plastic in 96 well plates.

4.3.3 Development of Analytical Methods for Liver Bioactive Targets

Because this project focussed on sheep liver, the bioactives extracted and amplified from the culture of liver cells yields sheep specific proteins. Unfortunately, there were not any detection products available from suppliers that could detect the sheep isoform of the protein. We therefore had to establish a method that was efficient at detecting the bioactive of interest and preferably able to quantify the amount of bioactive produced. ELISA was the optimal method however no kits for the detection of the sheep protein were available and alternative antibodies had to be sought to substitute into the assay to allow for the detection of the sheep product. In addition, there was no positive protein control available and we had to assess a number of samples that contained the target bioactive.

We also assessed a number of other methods for the detection and quantification of target bioactives from sheep liver cells. These methods included:

- RT-PCR
- ELISA
- 2-D gel Electrophoresis
- Other Analytical Methods

RT-PCR

In Milestone 5 we reported the development of an RT-PCR method that allowed for the detection of FGF2, IGF-2 and PDGF and transferrin mRNA from both liver and luteal cells. B-Actin was also detected and used as a PCR loading control to ensure that RNA and gel loading were constant. However the successful development of ELISAs for the detection of sheep FGF2 and PDGF resulted in this assay not being further used.

ELISA

An FGF2 ELISA kit was purchased from R&D Systems and we aimed to determine whether the kit could detect the bovine standard. The bovine standard was detected using the antibody combination provided in the kit and yielded a standard curve similar to that of the human FGF2 contained within the kit. To determine whether the FGF2 ELISA could detect the sheep isoform, we purchased a batch of sheep serum and used serial dilutions of this in an ELISA (Figure 10).

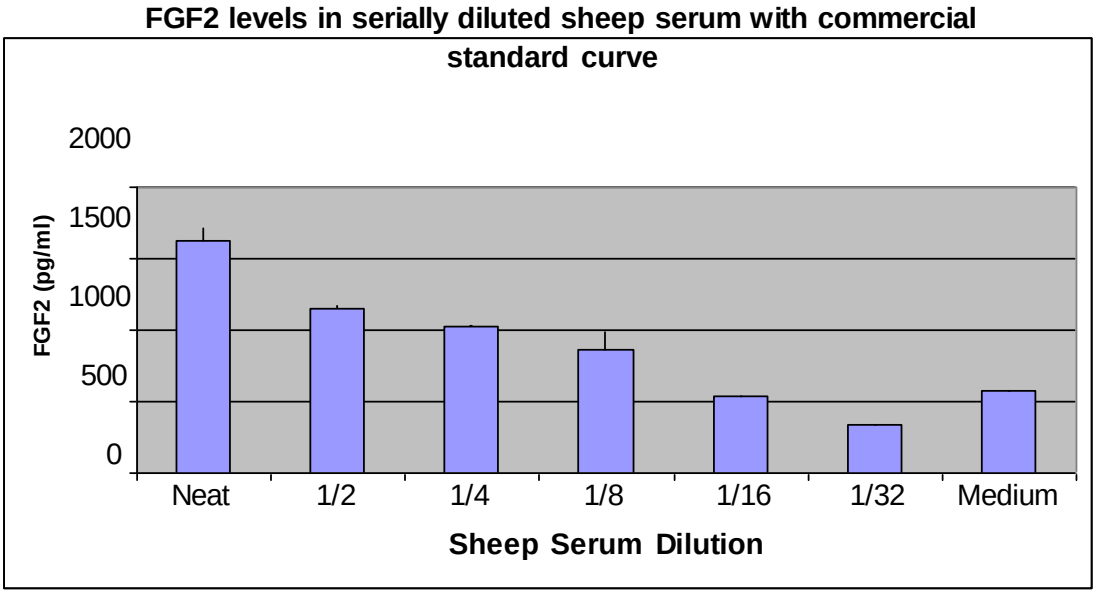


Figure 10. Serially diluted sheep serum and a hepatocyte conditioned medium samples were subjected to FGF2 ELISA. A standard curve was generated using kit capture and detection antibodies and bovine standard. The equation of the linear slope was calculated and FGF2 concentration from unknown samples was calculated. Data is presented as the mean + standard deviation of duplicate wells from 2 independent experiments.

Figure 10 demonstrates that sheep FGF2 can be detected in sheep serum and the amount of FGF2 detected correlates to the diluted serum sample. Neat serum contained more than 1600pg/ml and conditioned medium from unstimulated primary sheep liver cells demonstrated detectable levels. This data demonstrated the development of a sheep FGF2 ELISA.

The establishment of an ELISA to detect IGF-2 was initiated by the purchase of a mouse IGF-2 specific ELISA kit from R&D Systems. We found that the kit was unable to detect the human protein, indicating that it would be unlikely to detect the sheep isoform. Substitution of alternate commercial antibodies into the kit was unable to detect the human standard and therefore unlikely to detect the sheep protein.

The PDGF ELISA kit was purchased from R&D Systems, but was unable to detect any PDGF present in sheep serum. Upon substitution of the capture antibody with an abcam antibody (Rabbit polyclonal to PDGF-BB carboxyterminal end) resulted in a readable standard curve with the human standard PDGF indicating it may be suitable for the detection of sheep protein as the carboxy terminal end of PDGF is well conserved between species. When medium samples were subjected to the adapted ELISA, PDGF levels were measured that were above baseline levels, indicating that the PDGF ELISA could detect the sheep protein, demonstrating the successful development of an analytical method for the detection of PDGF.

The establishment of an ELISA for the detection of TNF-α was found to detect porcine standard when used with the human TNF-α ELISA kit purchased from R&D Systems. We found that this ELISA kit could detect TNF in the culture medium, however no measurable induction was observed with any stimulus, suggesting that further optimisation is required to generate a reliable assay.

2-D gel Electrophoresis

To use 2-D gel electrophoresis for the detection and quantification of target and unknown bioactives, we stimulated liver cells with retinoic acid for 48 hours or with a control and harvested the medium. To determine whether there was a large amount of protein present in the medium, we analysed the protein content by SDS-PAGE and Coomassie staining. We observed the presence of a large amount of protein within the sample and a strong band of approximately 60kDa, consistent with the size of albumin. To achieve the detection of low abundance proteins, albumin has to be reduced or removed from the sample. To accomplish this, we added ethanol to a final concentration of 42%, whereby albumin precipitates and can be removed by centrifugation. Upon completion of this step, we observed by SDS-PAGE that there was a large decrease in the amount of albumin in the sample, however further removal was required to enhance the detection of low abundance proteins in the sample (Milestone 5).

Other Analytical Methods

One method that we employed was Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF-MS). Four bioactive targets (Erythropoietin (Epo), Insulin-like growth Factor 2 (IGF-2), fibroblast growth factor 2 (FGF2) and tumour necrosis factor-α (TNF-α)) were detected by MALDI. IGF-2 was detected at a concentration of 500ng/µl and 5ng/µl (Milestone 4.2). A peak was observed at 7469.6, consistent with the molecular weight. An alternate peak was also observed at 14938.3, consistent with the mass to charge ratio of an IGF-2 dimer. Peaks of 3735.4 and 1857.9 were also detected and are consistent with IGF-2 protein with two and four positive charges, respectively (Figure 11).

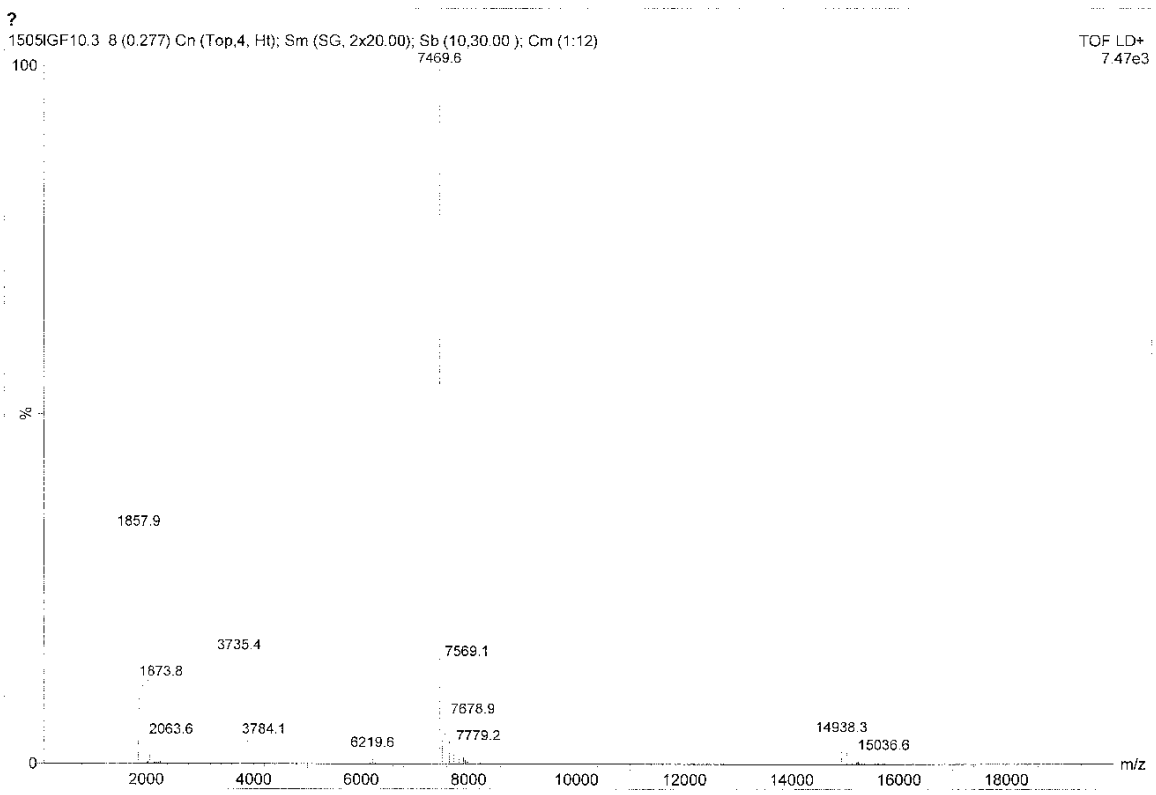


Figure 11. Mass spectrum of IGF-2 at a concentration of 500ng/µl
The stock solution of IGF2 was diluted to 500µg/ml and mixed with matrix. This solution was spotted onto a MALDI plate and analysed by MALDI-TOF-MS.

The TNF-α standard could be readily detected at 50ng/µl, producing a peak of 17164.1 Da, consistent with the manufacturer. FGF2 was also detected at the same concentration and yielded a peak of 16308.7 at 50ng/µl. Epo could not be detected by MALDI and none of the targets were detectable by SALDI.

The next step was to see if the standards could be detected in condition medium from primary sheep liver cell culture. Standards were spiked into the medium and analysed by MALDI. Five of the target bioactives could be detected (Table 2).

Target	Detected in Medium	Concentration (ng/ml)
IGF2	Conditioned cell medium	$1.6x10^3$
FGF-2	Conditioned cell medium	$2.5x10^4$
TNF-α	Conditioned cell medium	$2.5x10^4$
PDGF	Conditioned cell medium	$2.5x10^4$
Transferrin	Conditioned cell medium	$1.96x10^7$
Epo	Not detected	-

Table 2. Summary of the MALDI MS analysis and the concentration of the bioactive detected in the spiked conditioned medium.

4.3.4 Amplification of Bioactives from Liver Cells

The ability to induce bioactive production above levels currently harvested from animal organs or blood provides this technology with a competitive advantage. From the development of analytical methods for the detection of FGF2 and PDGF from sheep liver cells, the amplification of these two targets became the focus of this project. We demonstrated in Milestone 7 the amplification of FGF2 with multiple stimuli. The addition of PMA to the culture medium resulted in a 6-fold induction at concentrations of 50 and 200nM. This 6-fold amplification is consistent with data in the literature that shows similar amplification levels in Rat Muller Cells (Cheng et al. 1998) and 6-fold FGF2 promoter induction (Moffett et al. 1998). The addition of chemicals that mimic hypoxia (Desferrioxamine and cobalt chloride) both showed induction of FGF2 of 5-fold above baseline levels, consistent with published data demonstrating a 4-fold increase in rat cortical neurons (Sakaki et al. 1995). Other factors also shown to induce FGF2 expression were insulin by up to 8.5-fold and dexamethasone by 5.3-fold above control levels.

A number of stimuli tested showed a dose-dependent amplification of FGF2 expression. Calcium ionophore showed a maximal induction at the highest concentration tested (10^{-5}M) of 6.8-fold, consistent with a dose-dependent response reported in human fibroblasts (Sakai et al. 2001). FGF2 has been shown to be induced by retinoic acid by 2-3 fold at the mRNA level and up to 10-fold at the protein level in endothelial cells after four days of exposure (Gaetano et al. 2001). In this project, we demonstrated a dose dependent amplification of FGF2 with retinoic acid with a maximal induction of 9-fold after just 48 hours of culture. Arachidonic acid and glucose also induced FGF2 expression by up to 7.4-fold, higher than the 2-fold demonstrated in human microvascular endothelial cells (arachidonic acid) and peritoneal mesothelial cells (glucose) (Zeng et al. 2002; Teshima-Kondo et al. 2004; Ogata et al. 2001), indicating liver cells may be more efficient in responding to the stimuli. The addition of FCS to adhered liver cells resulted in an amplification of FGF2 by 20.3-fold with 20% FCS (Figure 12) which is much higher than previously observed levels of 2-fold and 4-5-fold in C6 glioma and smooth muscle cells, respectively (Powell et al. 1993; Winkles & Gay 1991).

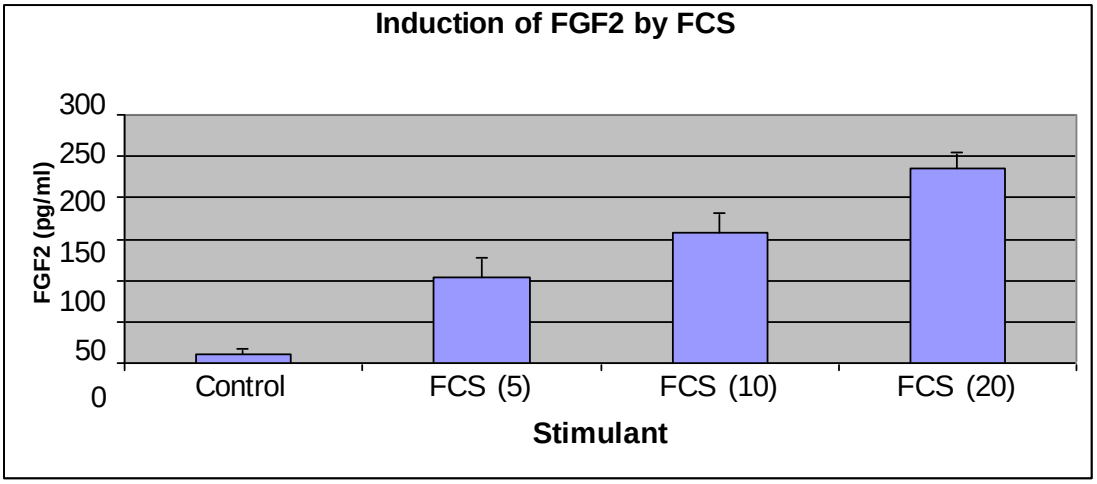


Figure 12. Freshly isolated sheep liver cells were stimulated with FCS (Foetal Calf Serum) at concentrations of 0 (control), 5, 10 or 20% for 48 hours. Medium was collected and subjected to FGF2 ELISA. Data is presented as the mean + standard deviation from replicate medium samples from 4 independent liver samples.

Similar to the amplification of FGF2 by a number of stimuli, the same samples were subjected to PDGF ELISA to determine whether the same stimuli could induce multiple bioactives simultaneously. Figure 13 shows the amplification of PDGF with retinoic acid of a dose-dependent response of 4.9-fold above control levels.

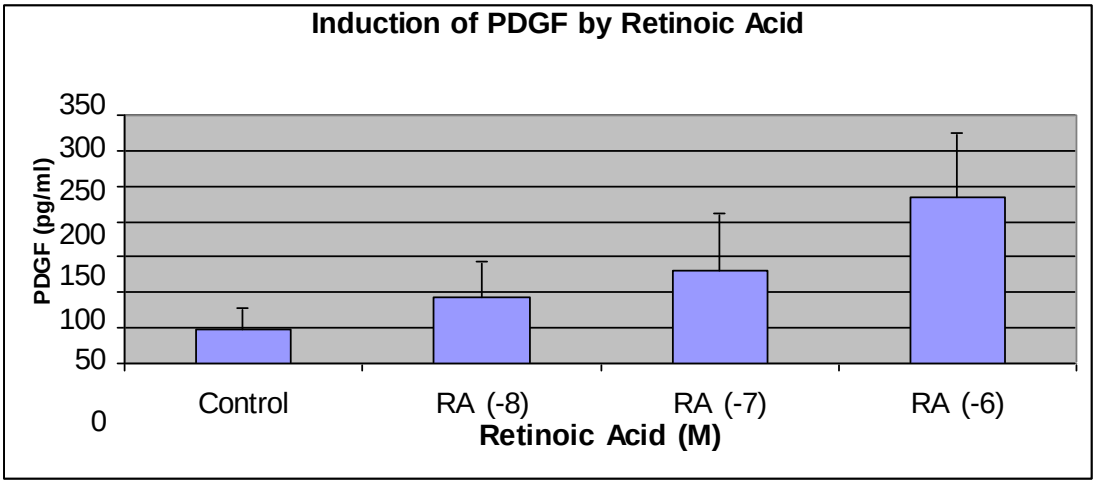


Figure 13. Freshly isolated sheep liver cells were stimulated with RA (Retinoic Acid) at concentrations of 0 (control) 10^{-8}, 10^{-7} or 10^{-6}M for 48 hours. Medium was collected and subjected to PDGF ELISA. Data is presented as the mean + standard deviation from duplicate medium samples from 4 independent liver samples.

Notably, the baseline levels of PDGF were much higher than that observed with FGF2 (compare 47 versus 16pg/ml) and although the fold-induction is lower than that observed with FGF2, the amount of protein measured is higher than that of FGF2 (compare 233pg/ml to 103pg/ml). PMA was shown to amplify PDGF expression by up to 3.9-fold in a dose-dependent manner, however FCS had a more modest effect, showing only a 2-fold increase at the optimal concentration of 5%. Glucose resulted in a 3-fold amplification at 20mM, but producing higher amounts of protein when compared to FGF2 levels. The addition of dexamethasone resulted in a dose-dependent response, yielding a 4.9-fold induction at 100nM. This demonstrates that several stimuli can induce the expression significantly above baseline levels and illustrates the ability to induce multiple bioactives from a sample stimulated with a single stimulus.

A summary of the maximal bioactive amplification observed is summarised in Table 3.

Stimulus	Optimal Concentration	FGF2 Amplification	PDGF Amplification
Phorbol Ester	200nM	6	3.9
Desferrioxamine	200nM	5	ND
$CoCl_2$	100nM	5	ND
Insulin	1000nM	8.5	ND
Dexamethasone	100nM	5.3	4.9
Calcium Ionophore	10^{-5}M	6.8	ND
Retinoic Acid	10^{-6}M	9	4.9

Arachidonic Acid	5µM	7.4	ND
Fetal Calf Serum	20%	20.3	2
Glucose	50mM	7.4	3

Table 3. Summary of the maximal fold amplification of FGF2 and PDGF from single stimuli. ND = not determined.

To determine whether the combination of stimuli could act additively or synergistically in the amplification of target bioactives, two stimuli were added to liver cells and bioactive levels analysed by ELISA. The addition of PMA to AA resulted in a synergistic amplification at the higher concentrations of PMA, whereas the addition of low glucose concentrations to PMA showed an additive effect (Milestone 9). Other stimuli combinations also showed additive or synergistic amplification of FGF2 production, however many combinations showed no further amplification that the addition of a single stimulus. Further optimisation of combining stimuli for target bioactive amplification will have to be repeated and assessed on different liver samples to determine which combinations result in the maximal amplification.

4.3.5 Scale-up of Liver Cell Culture

The disaggregation process of liver at a small laboratory scale has comprised of three main steps. Firstly, the liver is minced by a process of cutting the liver into approximately 1mm cubes using a scalpel blade. Secondly, the liver mince is placed into a 50ml conical flask with a collagenase solution (0.05%) and mixed by swirling at $37^{o}C$ for 2 hours, with cell collections every 30 minutes. Lastly, the cells are plated into tissue culture dishes which at laboratory scale have been 6 well plates, which have a surface area of $9.6cm^{2}$ per well.

To scale-up of the process of liver disaggregation involves modifying all three steps mentioned above. Steps one and two are closely linked as the measure of the efficiency and quality of these processes is assessed by the quality and number of cells that are recovered. The collagenase disaggregation step has been standardised to use 5ml of collagenase (0.05%) per one gram of tissue (See below). To economise the process we have found that the number and quality of cells collected is unaffected by the addition of up to 5 grams of tissue per 5ml collagenase. Therefore, when scaling-up the process, the ratio of collagenase solution to tissue was kept constant. To give an example, in this set of experiments, when scaling-up, the disaggregation was performed on 40g of tissue, meaning approximately 40 ml of collagenase solution was used in a 250 ml conical flask.

For the process of mincing the liver sample prior to collagenase disaggregation, we wanted to develop a method that could be adapted to a commercial scale. We sourced an electrical meat miner and compared the number of cells collected per gram of tissue with a number of attachments of the mincer to the small scale disaggregation protocol. We also compared a number of other large scale methods such as dicing the liver with a knife (similar to small scale) and the addition of protease inhibitors at low concentrations to prevent protein degradation (Figure 14).

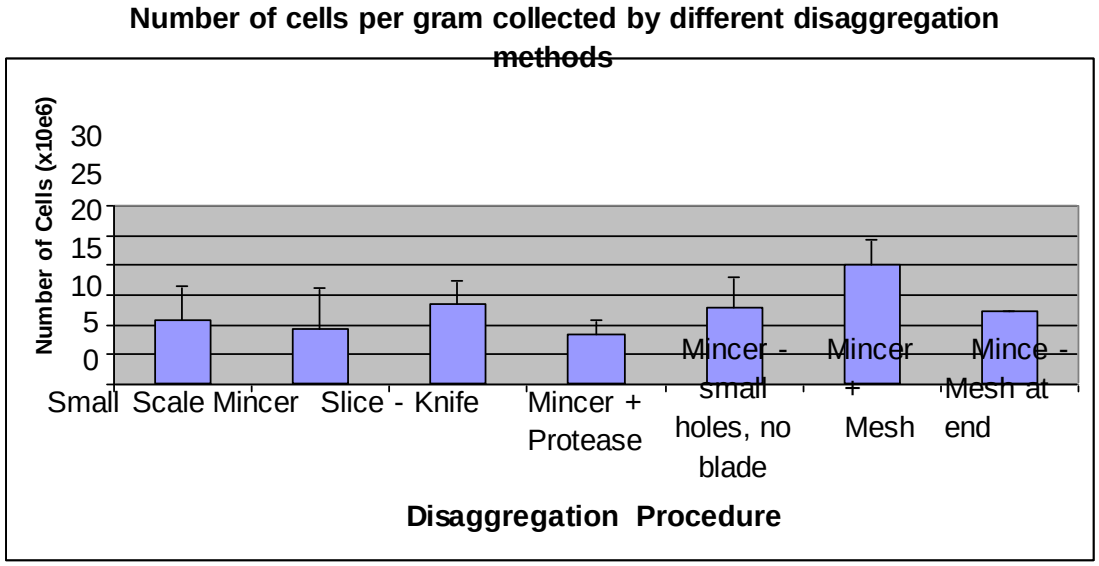


Figure 14. Liver tissue was disaggregated by the small scale method or in a mincer. The minced tissue was subjected to collagenase disaggregation and total number of cells counted post-disaggregation. The data is represented as the number of cells that were collected per gram of tissue. Data is presented as the mean + standard deviation from at least 3 independent experiments.

Figure 14 demonstrates that the number of cells collected was similar across each of the disaggregation methods. The quality of the cells was notably different between samples when compared to the small scale sample. Cutting the liver tissue with the knife produced lower quality cells when comparing the number of hepatocytes in the cell suspension and the amount of cell debris present. The mincer, particularly with the large holes, the use of protease inhibitors and cell collections with the mesh produced cells of similar quality to that of the small scale method. It was noted that the quality of the cells was relatively consistent between the different disaggregation methods and any differences were generally attributable to the liver sample. The mesh filter was extremely efficient at reducing the amount of collected liver tissue in the cell mix and suggests that to obtain a clean cell preparation, filtration is required.

The scale-up of liver cell culture to the 6 well (9.6cm^2 surface area) or into T25, T75 or T175 flasks was attempted using the conditions optimised for the 96 well format (0.09cm^2 surface area). Under these conditions, cell attachment was very poor or non-existent in the larger cell culture vessels, whereas attachment in the 96 well plates was consistent with previous findings. To optimise cell culture conditions in larger flasks, we coated flasks with cell attachment substrates such as gelatine, collagen and poly-L-lysine. Under all the conditions tested, cell attachment was poor, consistent with that observed with the uncoated flasks (Milestone 6).

To determine whether the FCS concentration affected the ability of the cells to adhere to the cell culture plastic, cells were plated into 96 well and 6 well plates. Consistent with previous findings, as the FCS concentration decreased, the number of cells attached to the 96 well plates also decreased as measured by MTT assay, indicating that serum is important for the attachment of cells in 96 well plates. When sheep liver cells were cultured in 6 well plates, the observation of cell attachment was inconsistent with that of the 96 well plates.

We sought to optimise the concentration of collagenase used in a scale-up process of cell plating. All work performed in 96 well plates was performed with a collagenase concentration of 0.25%, however scientific reports have indicated that lower concentrations may be more optimal for cell attachment. We found by Microscopy of 6 well plates that cells attachment was maximal with 0.05% collagenase concentration in serum free medium. To quantify the number of isolated liver cells, cells were trypsinised and counted by trypan blue exclusion assay (Figure 15).

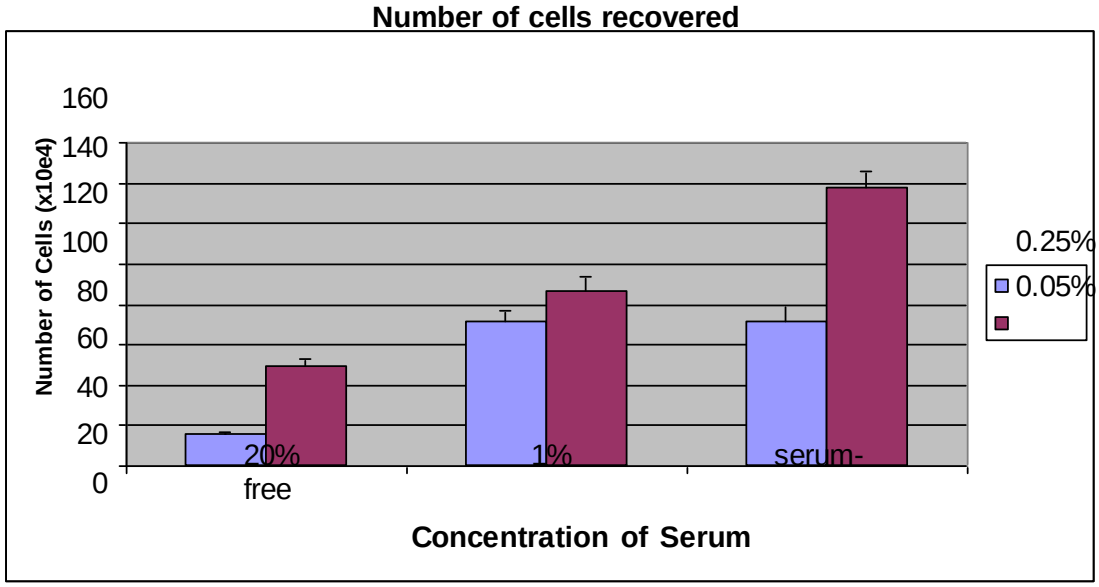


Figure 15. Sheep liver samples were disaggregated in either 0.25% (blue bars) or 0.05% collagenase (purple bars). 1.5 x 10^6 cells were seeded into each well of a 6 well tissue culture plate in different concentrations of FCS. 72 hours post-seeding, cells were subjected to trypsinisation and counted using a haemocytometer. Data is represented as the mean + standard deviation from 3 replicate cell counts.

Data presented in Figure 15 demonstrated that the number of cells present after three days in culture was higher for cells that were cultured in low serum. Furthermore, cells that were isolated with 0.05% collagenase yielded higher cell numbers. Therefore, this data indicates that serum-free HMM is the optimal medium for liver cell culture when the cells are isolated with 0.05% collagenase.

Upon the optimisation of the medium for plating in larger cell flasks, the large scale liver cell preparations were analysed to determine which method resulted in the maximal attachment. The attachment of the cells in T75 and T175 flasks demonstrated that the small scale and mincer sample with the mesh filtering had a large number of hepatocytes attached. The small scale samples and liver put through the mincer had the highest number of cells attached as determined by cell counting. However the level of attachment in normal cell culture flasks was quite low (between 30 and 50% of cells seeded) indicating that the use of an alternate cell culture flask treatment may promote better attachment.

Based on the number of cells collected from the large scale disaggregation methods, only samples put through the mincer were used with different size mince plates and all samples were collected with the mesh filter. To test whether liver cells could attach better to an alternative cell culture

treated plastic, cells were plated into normal and CellBIND flasks. Cells were plated into 6 well plates and allowed to adhere for 48 hours prior to trypsinisation and cell counting (Figure 16).

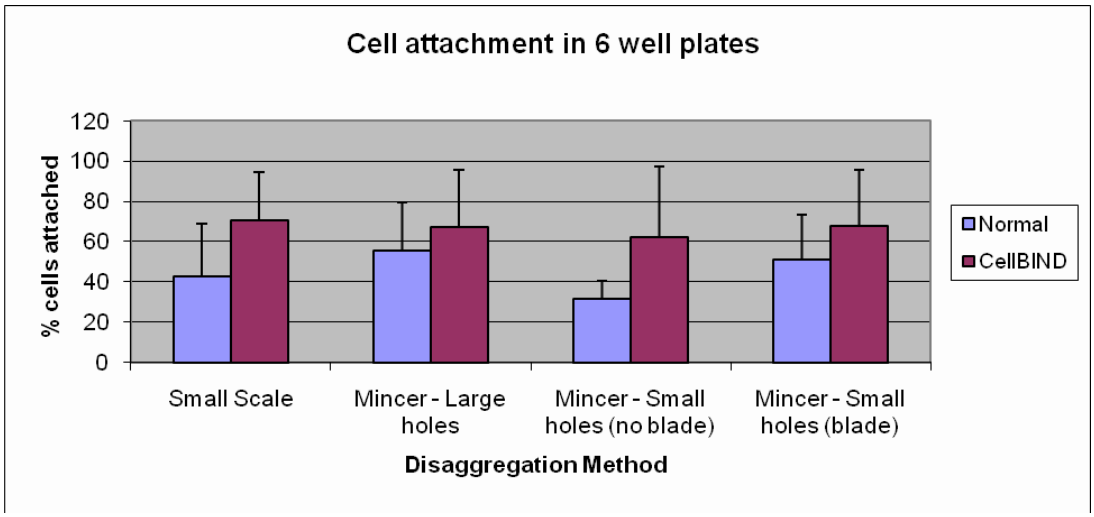


Figure 16. Liver cells were disaggregated by different methods and seeded into Normal (blue) or CellBIND (purple) 6 wells plates at a density of 2.5 x 10^6 cells per well. 48 hours after seeding, cells were trypsinised and counted. Data is presented as the mean + standard deviation from at least 5 independent experiments.

Figure 16 shows that the CellBIND flasks promoted better attachment under all of the disaggregation methods tested. The small scale and mincer with the large holes had the best attachment in the CellBIND flask (70% and 67% respectively) suggesting that the mincer with the large holes may be the optimal method for promoting the highest cell attachment to 6 well plates.

To determine whether the cells could attach efficiently to larger scale flasks, cells were plated into T7 flasks at a density of 1.56 x 10^7 cells per flask. Cells were plated into T75 flasks and allowed to adhere for 48 hours prior to trypsinisation and cell counting (Figure 17).

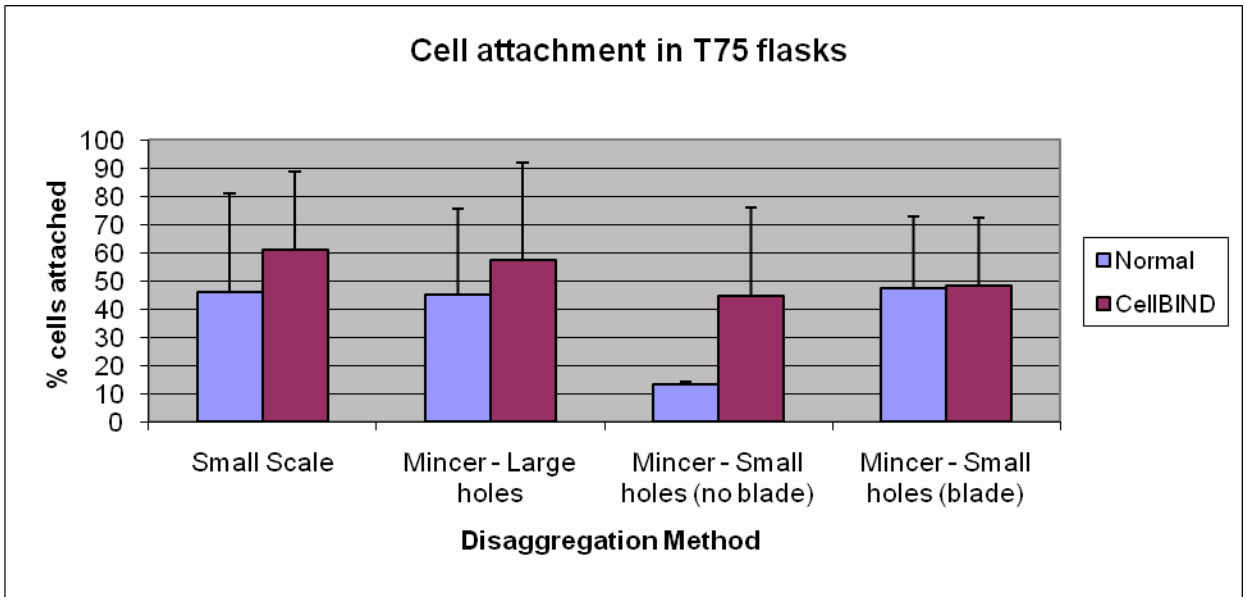


Figure 17. Liver cells were disaggregated by different methods and seeded into Normal (blue) or CellBIND (purple) T75 flasks at a density of 1.56 x 10^7 cells per flaks. 48 hours after seeding, cells were trypsinised and counted. Data is presented as the mean + standard deviation from at least 5 independent experiments.

Data presented in Figure 17 shows that the T75 CellBIND flasks promoted better attachment under all the disaggregation methods tested. In the CellBIND flasks, consistent with the 6 well dishes, the small scale and mincer with the large holes promoted the highest attachment (60.8% and 57.2% respectively) suggesting that the mincer with the large holes may be the optimal method promoting the highest cell attachment to T75 flasks in the CellBIND plastic ware.

To determine whether this data could be replicated in the largest sized tissue culture flasks available; cells were seeded into T175 flasks (surface area of 175cm^2) at a cell density of 3.65 x 10^7 cells per flask. Consistent with the 6 well dishes and T75 flasks, cells were seeded into normal and CellBIND flasks and allowed to attach for 48 hours prior to trypsinisation and cell counting (Figure 18).

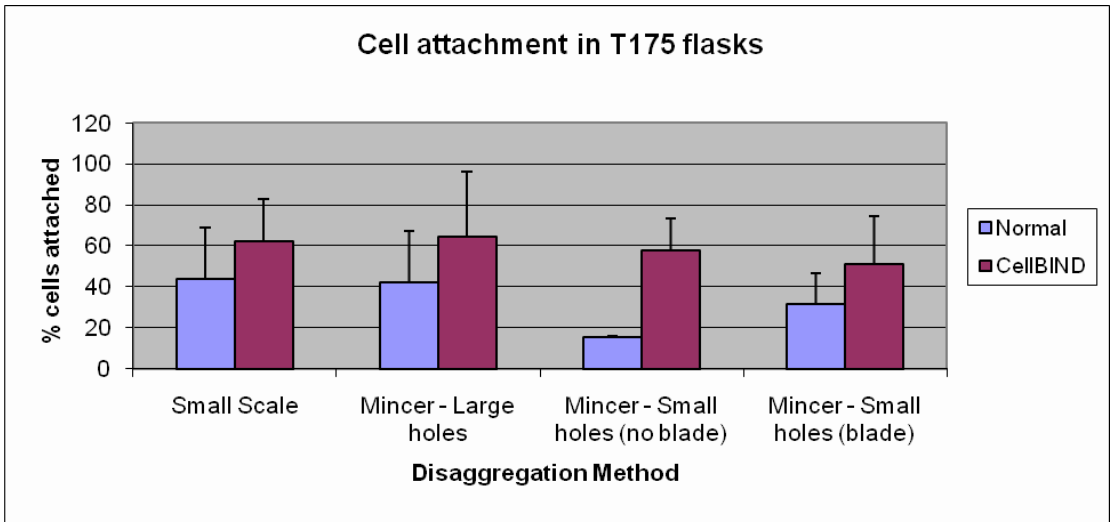


Figure 18. Liver cells were disaggregated by different methods and seeded into Normal (blue) or CellBIND (purple) T175 flasks at a density of 3.65 x 10^7 cells per flask. 48 hours after seeding, cells were trypsinised and counted. Data is presented as the mean + standard deviation from at least 5 independent experiments.

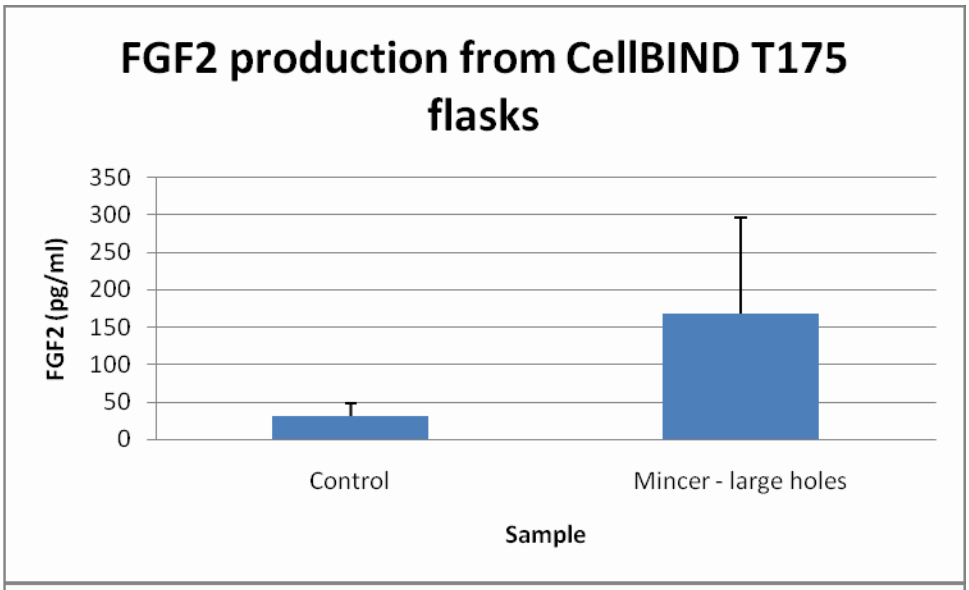
Figure 18 shows that the CellBIND flasks promoted better attachment under all the disaggregation methods tested, consistent with the 6 well dishes and T75 flasks. In the CellBIND flasks, the small scale and mincer with the large holes promoted the highest attachment (62% and 64% respectively) suggesting that the mincer with the large holes may be the optimal method promoting the highest cell attachment in the T175 flasks.

The cells that did not attach to the cell culture flasks were tested for cell viability 48 hours post-seeding and we observed that these cells were still viable indicating that it is possible that these cells may be able to produce bioactives. This will have to be assessed in the future and forms part of the proposal for the second phase of this project.

Based on the data presented in this report, it appears that the large scale disaggregation using the mincer with the large holes is the optimal method.

The aim of this project is to find a method that promotes optimal bioactive production in large scale. To determine which method promoted the optimal bioactive production, liver cells were stimulated with glucose at a concentration of 50mM as this stimulus was most efficient at amplifying both FGF2 and PDGF. Based on the disaggregation and plating data (Figure 16, 17 and 18 and Milestone 8 and 9), T175 flasks were assessed for FGF2 and PDGF amplification with samples disaggregated by the mincer with large holes only (Figure 19).

A.



B.

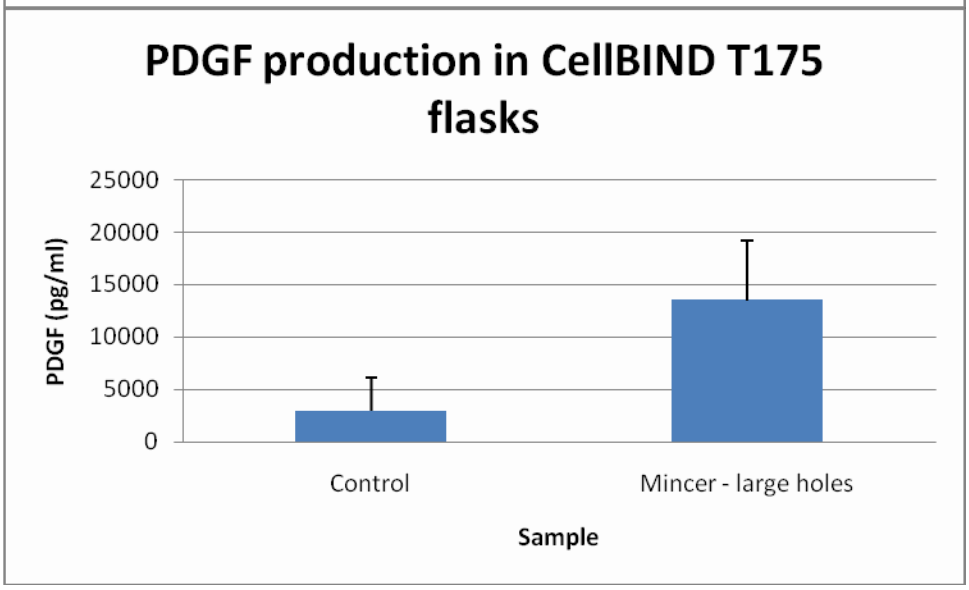
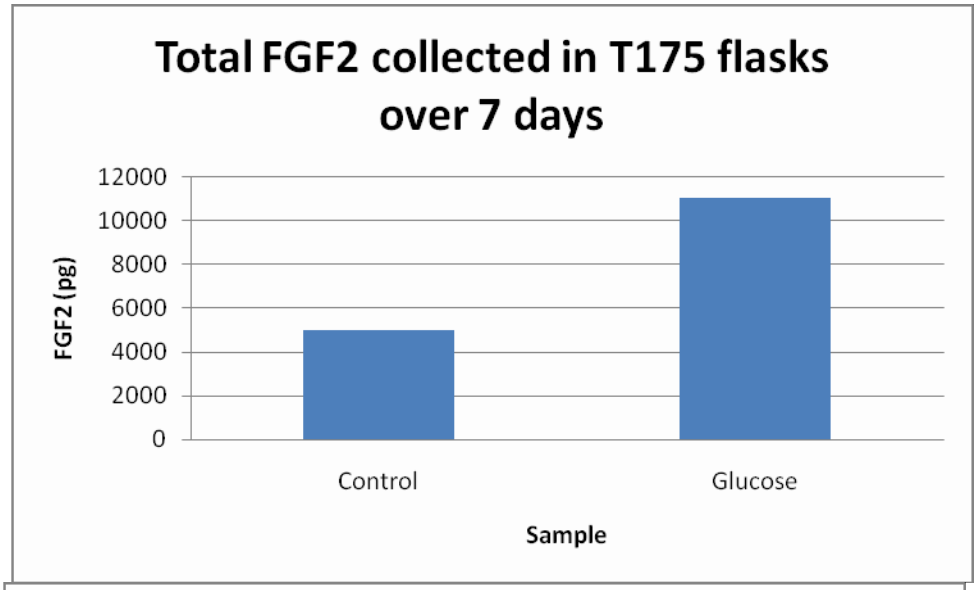


Figure 19. Liver cells were disaggregated and plated into CellBIND T175 flasks. Cells were subjected to glucose (50mM) for 48 hours and medium was collected (excluding control). **A.** FGF2 levels and **B.** PDGF levels were assessed by ELISA. Data is presented as the mean + standard deviation from at least 3 independent liver samples analysed in duplicate by ELISA.

Data in Figure 19 demonstrates the production of bioactives from CellBIND™ T175 flasks and the data indicates that both FGF2 and PDGF were amplified 5.4-fold and 4.5-fold, respectively above baseline levels. These data indicate that the large scale disaggregation methods employed and plating into large scale tissue culture flasks (T175) results in two target bioactives being amplified more than 4-fold.

We also wanted to determine whether the cells could be “milked” to produce more bioactive over a period of time. To determine whether the cells could be “milked” liver cells were seeded into T175 flasks and exposed to control or glucose (50mM) for 48 hours. Medium was collected four-times over a period of seven days. Bioactive production was assessed by ELISA and the total bioactive collected was calculated from the sum of 2 x T175 flasks (total of 320ml) (Figure 20).

A.



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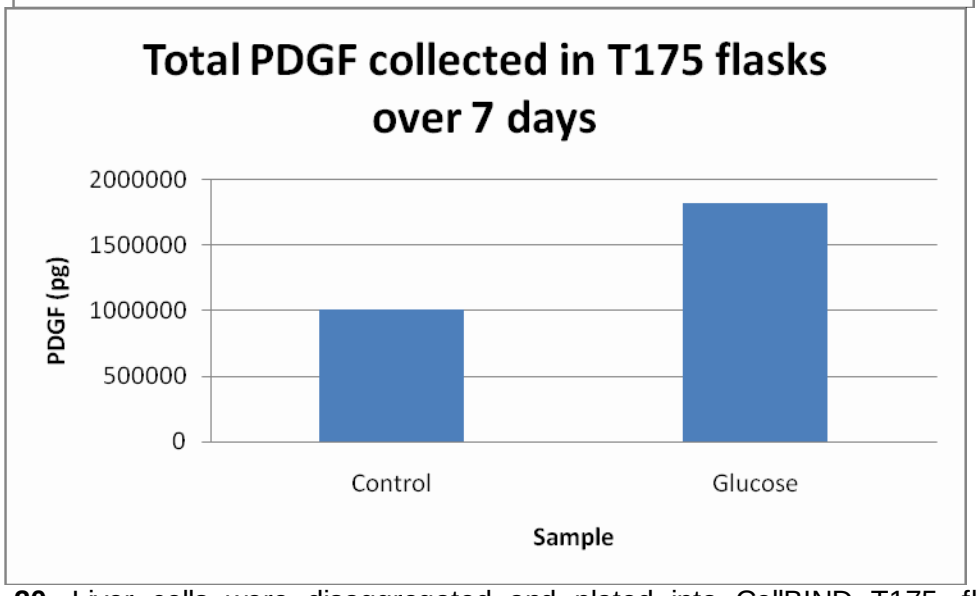


Figure 20. Liver cells were disaggregated and plated into CellBIND T175 flasks. Cells were subjected to glucose (50mM) and medium was collected every 48 hours over a period of one week **A.** FGF2 levels and **B.** PDGF levels were assessed by ELISA. Data is presented as the sum of the total bioactive collected from 2 independent T175 flasks with samples analysed in duplicate by ELISA.

Data presented in Figure 20 shows that the total amount of FGF2 collected from the control sample was more than 4000pg, however when stimulated with glucose, the levels could be increased to more than 10,000pg, resulting in more than a 2-fold increase. The production of PDGF was much higher than FGF2 in total bioactive, with more than 1,000,000 picograms (1µg) collected in unstimulated samples, and was increased to 1.8µg with the addition of glucose to the culture medium, giving a 1.8-fold amplification.

4.3.6 Integrated Process

The development of the integrated process for large scale disaggregation, cell isolation, plating and bioactive amplification is important to demonstrate that the process is robust and repeatable from organ collection to bioactive production. In Milestone 10 we demonstrated the successful implementation of an integrated process for the collection through to bioactive amplification from four independent liver samples and compared to the optimised small scale disaggregation samples.

The first stage of the integrated process was to measure the efficiency of the mincing and cell disaggregation method. This was measured by calculating the number of cells collected per gram of tissue that was disaggregated (Figure 21).

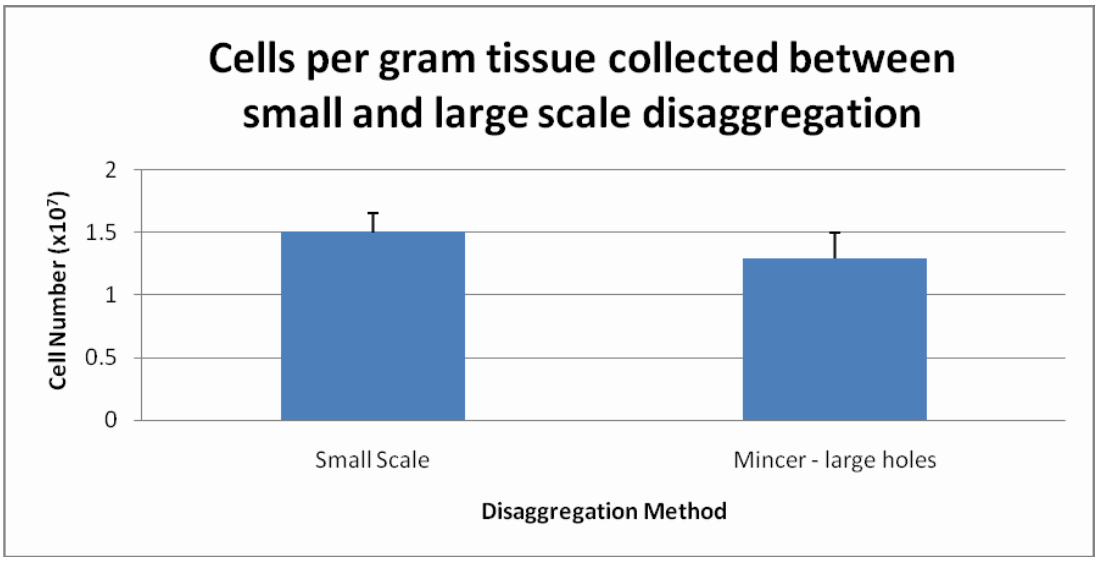


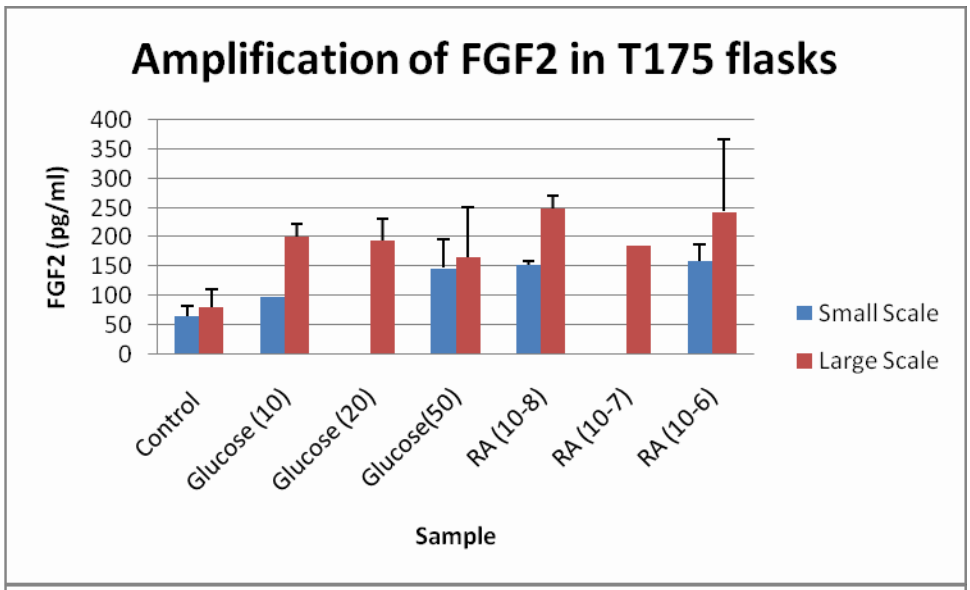
Figure 21. Approximately 30g of sheep liver tissue (from at least 3 independent sheep) was minced by either small scale methods (diced with a scalpel blade) or by large scale (mechanical mincing machine with large hole plate). Cells were disaggregated with collagenase and collected by centrifugation. Cells were counted under a haemocytometer and the number of cells per gram of tissue measured. Data is presented as the mean + standard deviation from 4 independent experiments.

Figure 21 shows that both methods of cutting up or mincing the liver tissue promoted efficient isolation of liver cells. When these cells were seeded into tissue culture flasks, it was noted that cell attachment was efficient (>90%) in the larger scale flasks, with the presence of many hepatocytes.

It was found that when the cells were stimulated with either glucose or retinoic acid at different concentrations in 6 well plates that it was observed that there was an amplification of FGF2 and PDGF in the samples. Both glucose and retinoic acid were shown to induce a higher level of FGF2 induction at the highest concentration tested when compared to the lowest concentration, suggesting a possible dose-dependent response. The amplification of PDGF was dose-dependnedt with both small and large scale liver preparations, with the largest induction of 13.8-fold observed with the small scale disaggregation with retinoic acid at 10^{-6}M. Large scale disaggregation showed the highest amplification at 20 and 50mM glucose of 11.5-fold above baseline levels.

The amplification of FGF2 and PDGF was tested under similar conditions in T175 flasks (Figure 22).

A.



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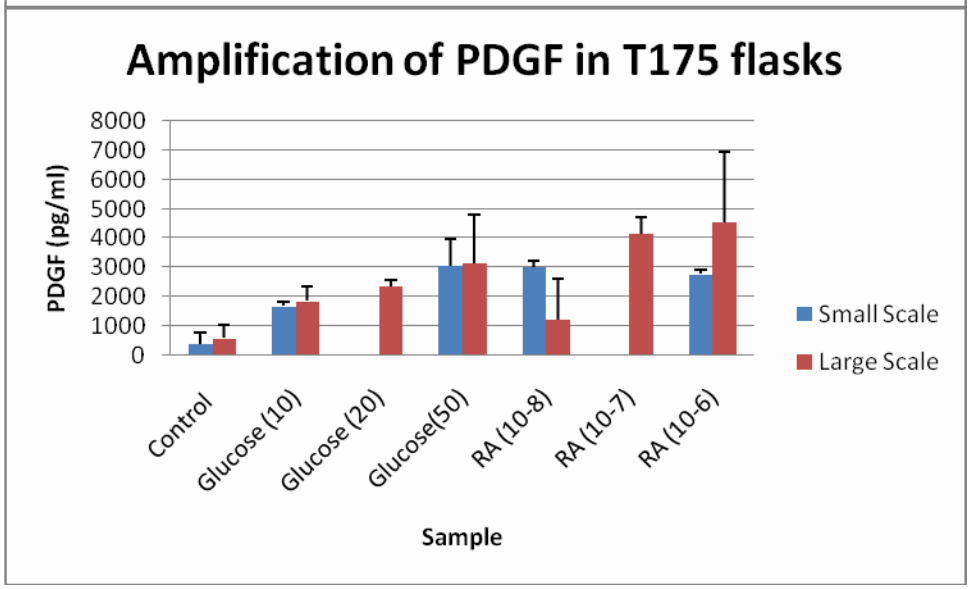

Figure 22. Liver cells were disaggregated by either small scale (blue) or large scale (red) methods as described above and plated into CellBIND T175 flasks. Cells were subjected to glucose (10, 20, or 50mM) or Retinoic Acid (10^{-8}, 10^{-7}, or 10^{-6}M) for 48 hours and medium was collected **A.** FGF2 levels and **B.** PDGF levels were assessed by ELISA. Data is presented as the mean + standard deviation from more than 4 independent liver samples analysed in duplicate by ELISA. Small scale disaggregation of glucose (20mM) and RA (10^{-7}M) were not determined.

All stimuli used in T175 flasks resulted in an amplification of bioactive production (both FGF2 and PDGF). The levels of induction were similar to that observed in T75 flasks (Milestone 10) showing higher fold induction levels with the cells disaggregated by the large scale mincer method. The highest fold induction of FGF2 was observed with RA at 10^{-8} and 10^{-6}M of 3.1-fold above baseline levels. PDGF amplification was maximal with RA at 10^{-6}M, showing a fold induction of 7.7-fold. These data demonstrate the achievement of amplifying bioactive production in large scale cell culture from a large scale liver disaggregation method.

5 Success in Achieving Objectives

5.1 General Achievements

For the success of this project, several tasks had to be completed prior to the commencement of researching bioactive amplification in the laboratory. For these tasks, we successfully completed the following:

- Set up a collaboration with T&R Pastoral Pty. Ltd. for organ collection
- All collection staff were vaccinated for Q-fever as part of the University's and T&R Pastoral's Occupational Health and Safety guidelines
- Provided commercialisation support with MLA and aided in the engagement of industry into the bioactives value-adding projects
- Completed a comprehensive literature and patent review that achieved the following:
    - Identified a number of target organs that synthesise bioactives
    - Identified potential bioactive targets synthesised in the corpus luteum and liver
    - Summarised the scientific literature on the isolation and primary culture of corpus luteum and liver cells
    - Identified the expression and amplification signals used to stimulate the expression of a number of potential bioactive targets synthesised in the corpus luteum or liver
    - Determined scalability of the process into a bioreactor and considerations of factors required to accomplish this task

5.2 Corpus Luteum

The successful demonstration of the amplification of a target bioactive from the corpus luteum aimed to provide proof-of-concept for this technology by utilising skills and techniques available within the Department.

For the corpus luteum, we successfully demonstrated the following:

- Collection and transport of ovaries from the meat processing plant to the laboratory in a sterile and viable manner
- The efficient disaggregation of cells from sheep corpus luteum using an established method
- Isolation of luteal cells from red blood cells using ficoll density gradients
- Removal of red blood cells from the culture was essential for the amplification of progesterone
- Survival and maintenance of cells *in vitro* for more than one week in culture
- Attachment of cells to cell culture dishes and plates
- Freezing and thawing of cells is a viable method for the availability of cells all year
- Frozen and thawed cells respond to external stimuli in a similar manner to freshly isolated cells
- Development of an ELISA for the detection and quantification of sheep progesterone
- Development of a HPLC method for the simultaneous detection of estrone, progesterone, 17-α pregnenolone and pregnenolone from complex media samples
- Freshly isolated and frozen/thawed cells have the ability to respond to external stimuli to amplify progesterone expression
- Progesterone expression can be amplified up to 10-fold with a single stimulus in sheep luteal cells
- Progesterone can be amplified more than 45-fold above baseline levels (and possibly up to 90-fold) with the combination of two stimuli
- Luteal cells can be cultured in large scale tissue culture format (more than a 1900-fold increase in surface area)
- Luteal cells cultured in large scale can be stimulated to amplify progesterone expression

5.3 Liver

The successful demonstration of the amplification of two bioactive targets simultaneously from liver aimed to provide proof-of-concept that this technology was achievable in two organs and that the basis of this technology could be used towards commercialisation.

For liver tissue, we successfully demonstrated the following:

- Collection and transport of liver from the meat processing plant to the laboratory in a sterile and viable manner
- Development of a novel, efficient and high-throughput liver disaggregation method
- Isolated liver cells can be maintained in a viable and sterile state for several days in culture
- Successful removal of red blood cells from the culture using slow centrifugation protocols
- Consistency in disaggregation between liver samples
- Successful development of analytical methods for the detection of two target bioactives (Fibroblast growth factor-2 and platelet-derived growth factor) by ELISA
- Identification of a number of stimulants for amplification of FGF2 and PDGF synthesis
- The ability to amplify FGF2 20.3-fold above baseline levels
- The ability to amplify PDGF 4.9-fold above baseline levels
- Dual amplification of FGF2 and PDGF using the same stimulus with more than five different stimuli

- The ability to collect, mince, disaggregate and culture liver cells in an adaptable large scale format
- The ability to amplify bioactive production in a large scale format
- Integration of the process from organ collection through to bioactive amplification in sheep liver cells in a large scale format from four independent sheep liver samples

6 Impact on Meat and Livestock Industry – Now & in Five Years Time

This project has demonstrated that the amplification of bioactives from *in vitro* cultured primary cells derived from farmed animal organs is feasible. The successful culture and amplification of bioactives in T175 flasks suggests that the commercialisation of this process in larger cell culture flask systems could be implemented when a commercially viable target protein is identified. The identification of a commercially viable target bioactive that has a route to market is of paramount importance for the successful uptake and implementation of this technology by industry. The target bioactive will require a market size and economic viability study to determine the best target bioactive. This market and industry research will be conducted in the next phase of the project in conjunction with MLA. Developing the process for such a target bioactive will result in this technology being a more attractive investment for industry.

Successful implementation of the technology to the target bioactive(s) will result in this process being ready for commercialisation within one to two years. The uptake of this technology by industry will add value to the meat processing industry via product diversification and to the bioactive producers by offering competitive advantages of price, quality and supply volumes over competitors.

Within five years, we envisage that this technology will be in commercial production. The production of bioactives from animal organs could be sourced from either a less technical cell culture process producing bioactives from a mixture of animal cells for subsequent purification (to be assessed in the next phase of the project) or a high technology cell culture process that would be modified, optimised and scaled-up to a commercial scale, producing targeted bioactives form isolated cell cultures.

7 Conclusions and Recommendations

This project has demonstrated a proof-of-concept of the feasibility of amplifying bioactive production in culture of primary cells derived from farmed animal organs. This project has demonstrated for two organs (corpus luteum and liver) of sheep the:

- Collection and transport of organs
- Disaggregation and collection of specific cell types from the collected organs
- Maintenance and culture of cells *in vitro*
- Amplification of bioactive targets using cost-effective stimuli

- Scalability of the process from small laboratory scale (surface area of 0.09cm^2) to large laboratory scale tissue culture flasks (surface area of 175cm^2)

Industry have communicated that they require a commercial process within one to two years of investment. To enable a fast-track to commercialisation, several key tasks must be completed to enable the success of this technological opportunity:

- Identification of marketable and economically viable target bioactive or bioactives
- Feasibility study of less technical cell culture method for bioactive amplification
- Feasibility study on the transfer of technology to bovine organs
- Scalability of the process from tissue culture flasks to a bioreactor system

The identification of a target bioactive that has a quick route-to-market and is economically viable with a good market size is critical for the commercialisation of this technology. We recommend as part of the next phase of this project working closely with MLA in the development of a Market Size and Feasibility Study to aid in the identification of a target bioactive and assist in commercialisation of this product to market. This bioactive target will require the development of analytical methods for the detection and quantification from ovine and bovine sources.

We recommend the transfer of the technology generated in the first phase of this project to bovine organs. The major reason for this is that there are a lot of bovine bioactive products on the market, particularly in the medical and scientific field. Sheep (ovine) products are less common and therefore it may be an easier route-to-market with a bovine bioactive product. We plan to begin the transfer of the technology developed in sheep liver to cow liver to demonstrate the ability to disaggregate, culture and amplify bioactive targets from these cells, hence the requirement for the development of analytical methods for the detection of bovine bioactives.

We recommend determining the feasibility of a less technical method, involving mincing or slicing liver tissue and culturing the liver *in vitro* for the amplification of bioactive production. This process, if successful, is expected to be more attractive to an investor as it speeds up the commercialisation.

Finally, we recommend that the technology developed during the first phase of this project be tested for scalability into a bioreactor system for the mass production of target bioactives. Demonstration in a small bioreactor will prove the scalability of the process and this, in conjunction with the optimisation of amplification signals in plates, will show the potential of this technology.

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9 Appendices

9.1 Research Operating Procedures

Research Operating Procedure 1: T&R Protocols

1. Enter T&R at second gate with stone wall.
2. Press button for entry.
3. Park in visitor's car park – directly to the right.
4. Go to the main office and sign in.
5. Ask receptionist for a visitor's coat, boots, ear protection and hair net.
6. Return to the car to collect equipment and esky.
7. Leave all valuables in the car hidden in car (Jewellery not permitted in meat processing room).
8. Go to the entrance to the meat processing floor.
9. Wash boots first.
10. Wash hands with green soap, followed by blue ethanol wash.
11. Go to collection area.
12. Upon leaving, wash boots and hands with green soap and blue ethanol wash.
13. Return boots and coat to Reception.
14. Discard hair net and ear protection in the bin.
15. Sign out at Reception.
16. Press button to speak to receptionist to leave.

Research Operating Procedure 2: Collection of Animal Tissue

1. Sterilise equipment several days prior to sample collection by autoclaving:
    a. Scissors (x2)
    b. Scalpel blades (several)
    c. Tweezers (x2)
    d. Collection pots
2. Prepare sterile solutions for the collection of tissue:
    a. Liver - RPMI medium + antibiotics
    b. Corpus Luteum - HBSS medium + antibiotics
3. Preparation of visit – Collection of equipment needed:
    a. Sterilised equipment
    b. Gloves – several pairs each
    c. Paper towel
    d. 30 ml or 200 ml sample pots
    e. Collection tray
    f. Ethanol Spray
    g. Tissues
4. Sample pots require the addition of 10 ml of RPMI or HBSS per 30 ml collection tube or 150 ml per 200 ml sample pot.
5. Set up number of sample pots per tissue samples required.
6. Sample pots containing RPMI or HBSS must be stored on ice at 4^0C at least overnight to ensure the collection solution is evenly chilled.
7. On the day of sample collection, fresh ice is placed into an esky containing the sample pots and sealed with the esky lid.
8. At T&R Pastoral, follow Research Operating Procedure 1: T&R Protocols.
9. Upon entering the meat processing room, alert the line manager of the organ you wish to collect.
10. Collect the organ directly from the line in a pre-ethanol sprayed and wiped container.
11. Cut approximately 1-2 gram segments of tissue from the organ and place directly into the sample pot containing ice-cold RPMI (liver) or HBSS (Corpus Luteum), opening the lid for as little time as possible.
12. Return the unused organ to the line and rinse container under tap.
13. Transport samples on ice to the laboratory.

RPMI
To make one litre add the following:
RPMI 1640 powder 10.40g/L (Thermo 50-020-PC)
Sodium bicarbonate solution 17.8ml/L (Pfizer DW99)
Penicillin / Streptomycin 10ml/L (Thermo SV30010)
Adjust pH to 7.2

HBSS
To make one litre add the following
1 bottle of HBSS powder (Sigma H1387)
Sodium bicarbonate solution 4.1ml/L (Pfizer DW99)
Phenol Red 0.011g/L (Sigma P3532)

Penicillin / Streptomycin 10ml/L (Thermo SV30010)
Adjust pH to 7.2

Research Operating Procedure 3: Disaggregation of Corpus Luteum

1. Place the collected Corpus luteum (CL) into a sterile biohazard cabinet
2. Excise the CL by slicing the CL through the centre with a sterile scalpel blade and push it away from the surrounding tissue into a sterile petri dish
3. Slice the CL as thinly as possible using a scalpel blade and place into a pre-weighed, sterile 50ml conical flask
4. Record the exact weight of CL
5. Add 10ml of 400U/ml Type II Collagenase (Invitrogen 17101015) per gram of tissue
6. Incubate at 37^{O}C for 40 mins with shaking
7. Remove the collagenase from the CL tissue and replenish with fresh collagenase at 400U/ml
8. Incubate for 2 hours at 37^{O}C with shaking
9. Collect cells using a transfer pipette to a 10ml centrifuge tube and pellet cells at 200xg for 5 mins.
10. Wash cells with DMEM medium containing 10% FCS and pellet cells at 200xg for 5 mins.
11. Repeat Step 10, for a total of two washes.
12. Store cells on ice until ready to plate.

DMEM
Follow the Instructions on the DMEM bottle to make up one litre of medium
To the medium, add:
Sodium bicarbonate solution 17.8ml/L (Pfizer DW99)
Sodium pyruvate 2.2g/L (Sigma P5280) L-
glutamine 0.5g/L (Sigma G8540)
Penicillin / Streptomycin 10ml/L (Thermo SV30010)

Research Operating Procedure 4: Isolation of Luteal Cells

1. Luteal cells are isolated by using a ficoll density gradient
2. Place 3ml of lymphoprep (ficoll – GE Healthcare 17-1440-03) into a 10ml centrifuge tube
3. Carefully layer 2ml of luteal cell suspension on top of the ficoll layer
4. Centrifuge the density gradient at 1500rpm in a benchtop centrifuge for 5 minutes
5. Carefully remove the cell layer and transfer to a fresh centrifuge tube
6. Add 10ml DMEM containing 10% FCS to the centrifuge tube and pellet the cells at 150xg for 5 mins.
7. Repeat Step 6 for a total of 2 washes
8. Resuspend the cell pellet in 2ml medium and perform cell counts (See Research Operating Procedure 5: Cell Counts using a Haemocytometer).

DMEM
Follow the Instructions on the DMEM bottle to make up one litre of medium
To the medium, add:
Sodium bicarbonate solution 17.8ml/L (Pfizer DW99)
Sodium pyruvate 2.2g/L (Sigma P5280)
L-glutamine 0.5g/L (Sigma G8540)
Penicillin / Streptomycin 10ml/L (Thermo SV30010)

Research Operating Procedure 5: Cell and Viability Counts using a Haemocytometer

1. Take a 20µl sample of cell suspension and place into a clean well of a 96 well tray
2. Clean the surface of the haemocytometer with 70% ethanol and dry with Kleenex tissue
3. Mix 20µl of trypan blue solution (Sigma T6146) with the cell sample and mix thoroughly
4. Collect 20µl of sample into a yellow pipette tip
5. Transfer the cell suspension to the edge of the haemocytometer chamber and expel the suspension and allow it to be drawn under the coverslip by capillarity. IMPORTANT – Do NOT overfill or underfill the chamber.
6. Count all cells in at least 2 large squares of a haemocytometer depending on the number of cells present.

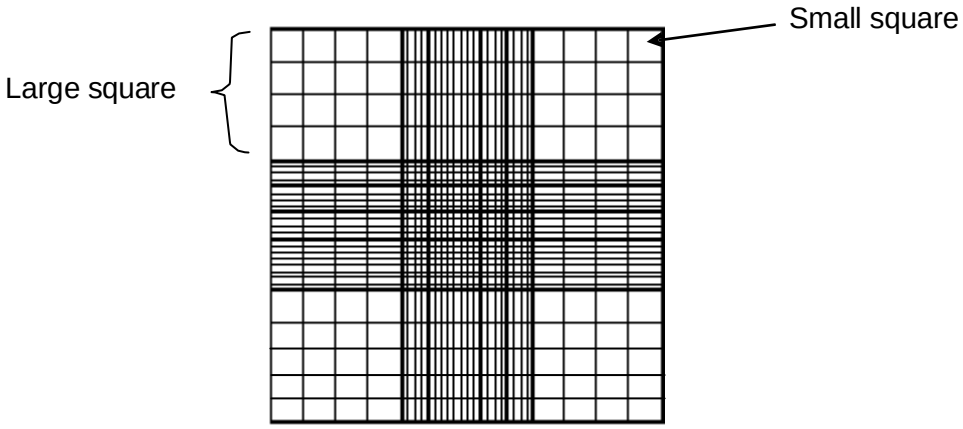

Figure 1. View of a haemocytometer under a microscope. (Figure modified from http://149.170.199.144/new_rd/contents/poisson.htm)

7. Calculate the number of cells by dividing the number of cells counted by the number of squares counted.
8. Multiply the number calculated in step 7 by the dilution factor (x2)
9. Multiply the number of cells calculated in step 8 by 1×10^4
10. This number is the number of cells present in 1ml of sample
11. Count the blue cells (dead) in the same square as total cell counts
12. Calculate viability by subtracting the number of dead cells counted from the total number of cells counted and divide the answer by the total number of cells counted. Express the answer as a percentage of total cells counted. Note: This count is based on numbers counted under the haemocytometer, not on the calculated number of cells per ml of sample

Research Operating Procedure 6: Plating of Luteal Cells into Cell Culture Flasks

1. Luteal cells are to be plated into DMEM containing 10%FCS and antibiotics
2. To seed cells into 96 well trays, cells are plated at densities from 10,000 to 20,000 cells per well in 200µl of medium
3. To seed cells into 6 well trays, 6×10^5 cells per well into 1.5ml medium
4. To seed T25 flasks, 1.67×10^6 cells per flask added to 5ml medium
5. To seed T75 flasks, 5×10^6 cells per flask added to 12ml medium
6. To seed T175 flasks, 1.17×10^7 cells per flask added to 40ml medium
7. Incubate cells in a CO_2 incubator at 37^oC and 5%CO_2 overnight to allow cells to adhere to the tissue culture dish or flask

DMEM

Follow the Instructions on the DMEM bottle to make up one litre of medium
To the medium, add:
Sodium bicarbonate solution 17.8ml/L (Pfizer DW99)
Sodium pyruvate 2.2g/L (Sigma P5280) L-
glutamine 0.5g/L (Sigma G8540)
Penicillin / Streptomycin 10ml/L (Thermo SV30010)

Research Operating Procedure 7: Stimulation of Progesterone from Sheep Luteal Cells

1. Prepare the stimulatory factors by dissolving them in the appropriate solvent (Luteinizing hormone – MQ water; 22-Hydroxy Cholesterol – ethanol; dibutyryl cyclic-AMP – MQ water).
2. Calculate the volume of medium required for each treatment and prepare a mastermix of each stimulant at the desired concentration in DMEM containing 10% FCS.
3. Concentrations of each stimulant should be in the following range:
    a. Luteinizing hormone (Sigma L5269) – 10-100ng/ml
    b. 22-Hydroxy Cholesterol (Sigma H9384) – 100 – 1000µM
    c. Dibutyryl cyclic-AMP (Sigma 34988) – 0.1 – 10mM
4. Remove luteal cells from the incubator that have adhered overnight following Research Operating Procedure 6: Plating of luteal cells into cell culture flasks.
5. Label the wells or flasks with the concentration and stimulant added
6. Aspirate the medium from the wells or flasks
7. Carefully add the medium containing the correct concentration and stimulant to each well / flask
8. Return the trays or flasks to the incubator and incubate the cells at $37^{O}C$ and 5%CO_2 for 48 hours.
9. Collect the medium and transfer to a freshly labelled 96 well tray or 10ml centrifuge tubes and store at $-20^{O}C$ until ready to assay.

Research Operating Procedure 8: Analysis of Progesterone by ELISA

Day 1

Plate Coating

1. Use NUNC Maxisorp plates (44-2404 - Level 4 compactus)
2. Dilute primary progesterone antibody (Rabbit anti-progesterone) 1:20,000 in coating buffer
3. Add 50µl per well of antibody to plate
4. Tap plates gently to ensure that the coating covers the bottom of the well
5. Label, cover with acetate plate sealer and incubate overnight at $4^{O}C$ (at least 12 hours)

Day 2

Standards

1. Standards used are 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.12ng/well.
2. Dilute standard working stock (800ng/well or 16ng/ml) serially (2-fold) by using 200µl stock plus 200µl cell culture medium

Samples

1. Prepare media samples by diluting (as required) in medium to an appropriate dilution (approximately 1:100 to 1:1000)

HRP

1. Progesterone HRP conjugate working dilution is 1:40,000
2. Add 25µl of HRP working stock (1:200) to 5ml of EIA buffer to make the working dilution. Store this solution on ice.

Plate Washing

1. Wash the plate 5 times with wash solution
2. Blot the plate on paper towel to remove excess wash solution

Plate Loading

1. Pipette 50µl of standard or sample per well as quickly and accurately as possible, following the plate map
2. Add 50µl of diluted Progesterone HRP to all wells that contain standard, control or sample. Avoid Splashing!
3. Note: No more than 10 minutes should pass during this process.
4. Cover plates with acetate plate sealer and incubate at room temperature for exactly 2 hours

Plate Washing

1. Wash the plate 5 times with wash solution
2. Blot the plate on paper towel to remove excess wash solution
3. Plates are fairly stable at this point and can be left upside down on bench top until all plates are washed (no longer than 1 hour).

Substrate

1. Prepare ABTS substrate immediately before use (within 20 mins)
2. Combine 40µl 0.5M H_2O_2, 125µl 40mM ABTS and 12.5ml substrate buffer and mix well
3. Add 100µl ABTS substrate to all wells that contain standard, control or sample
4. Cover with plate sealer and incubate at room temperature with shaking
5. Plate colour development will vary based on the age of HRP and/or antibody, but should be less than one hour.

Plate Reading

1. Optical density (OD) of 0 wells should read 1 or less
2. Optimal readings for 0 wells: >0.7 to <1.0 OD.
3. Read plate at 405nm (reference 540 nm)

Progesterone Stock Preparation

Antibody

1. Dilute primary antibody (CL425) at a dilution of 1:50 by adding 100µl of stock to 4.9ml of coating buffer
2. Aliquot 300-400µl into vials and store at -20^{o}C.
3. Store Antibody stock at -80^{o}C.

HRP Conjugate

1. Dilute progesterone-3CMO-horseradish peroxidise 1:200 by adding 25µl of stock to 4.975ml EIA buffer for a working stock and store at 4^{o}C.
2. Store HRP stock at -80^{o}C.

Standards

1. Weigh out 0.5mg of progesterone (Sigma Cat. # P0130) and add 5ml ethanol in a scintillation vial for stock solution of 100µg/ml.
2. Dilute the 100µg/ml primary stock 1:5 by adding 1ml to 4ml ethanol for a 20µg/ml secondary stock (1000ng/well). A well is equal to 50µl.
3. Dilute the 20µg/ml secondary stock 4:5 by adding 4ml to 1ml ethanol for a 16µg/ml (800ng/well) working stock.
4. Aliquot working stock and store all stocks at -20^{o}C.

Assay Buffers

Coating Buffer

0.795g Na_2CO_3 (Anhydrous) (Sigma S2127)
1.465g $NaHCO_3$ (Sigma S7561)
H_2O to 500ml

pH to 9.6 and store at 4^oC.

EIA Buffer

Stock A Solution (0.2M NaH_2PO_4)

13.9g NaH_2PO_4 (Sigma S9638)
500ml H_2O

Stock B Solution (0.2M Na_2HPO_4)

14.2g Na_2HPO_4 (Sigma S7907)
500ml H_2O

97.5ml Stock A
152.5ml Stock B
4.35g NaCl (Sigma S9625)
0.5g BSA (Sigma A7906)
250ml H_2O

pH to 7.0 and store at 4^oC

10x Wash Solution

43.83g NaCl (Sigma S9625)
2.5ml Tween 20 (Sigma P1379)
500ml H_2O

Store at 4^oC

Dilute 10-fold for working wash solution (100ml wash solution plus 900ml H_2O). Store at room temperature

Substrate Buffer

4.805 g Citric Acid (Sigma C0759)

pH to 4.0, store at $4^{o}C$.

ABTS (40mM)

1.55 g ABTS (Sigma A1888)
pH to 6.0
Make up to 25ml H_2O total volume

ABTS is light sensitive – use brown glass or foil for storage at $4^{o}C$.

Hydrogen Peroxide (0.5M)

500µl 30% H_2O_2 solution
8ml H_2O

Store at $4^{o}C$

Research Operating Procedure 9: Freezing and Thawing of Sheep Luteal Cells

Freezing of Cells

1. Collect sheep ovaries by following Research Operating Procedure 2: Collection of Animal Tissue.
2. Disaggregate the corpus luteum by following the Research Operating Procedure 3: Disaggregation of the corpus luteum.
3. Isolate luteal cells following Research Operating Procedure 4: Isolation of luteal cells.
4. Calculate the number of cells collected following Research Operating Procedure 5: Cell and viability counts using a haemocytometer.
5. Prepare the freezing medium and place on ice
6. Pellet the cells at 1000rpm in a benchtop centrifuge for 5 minutes
7. Aspirate the medium and resuspend the cells in freezing medium to a concentration of 1 x 10^6 cells/ml.
8. Place 1ml of cell suspension into a labelled cryotube, secure the lid and place immediately on ice.
9. Incubate the cells in a -20^{O}C freezer for 1 hour
10. Transfer the cells to a -80^{O}C freezer and incubate overnight
11. Transfer the cells to liquid Nitrogen the next day

Thawing of Cells

1. Collect cryovial from liquid Nitrogen storage and place cells on ice
2. Transfer cells to tissue culture laboratory
3. Thaw cells as quickly as possible in a 37^{O}C water bath
4. Once the cells are liquid, add 1ml of DMEM containing 10% FCS to the cells dropwise
5. Transfer the cells to a 10ml centrifuge tube and add medium to 10ml total volume
6. Pellet the cells by centrifugation at 100 x g for 5 mins
7. Resuspend the cells in 1ml DMEM containing 10% FCS and count the cells using Research Operating Procedure 5: Cell Counts using a haemocytometer
8. Plate the cells into the desired tissue culture dishes / flasks following Research Operating Procedure 6: Plating of luteal cells into cell culture flasks

Freezing Medium:

Prepare DMEM as per Research Operating Procedure 6: Plating of luteal cells into cell culture flasks
To the DMEM add
FCS to 40%
DMSO (Sigma D2650) to 10%
Store on ice

Research Operating Procedure 10: MTT Assay

1. Plate cells into 96 well tissue culture trays
2. Add MTT (Sigma M2003) to a final concentration of 0.5 mg/ml (stock @ 5 mg/ml)
3. Incubate cells in MTT for 24 hours
4. Add 80µl per well of 20% SDS (Sigma L4390) in 0.2M HCl
5. Mix with a pipette gently
6. Ensure there are no bubbles present in each well as they will interfere with the spectrophotometer readings
7. Incubate plate in the dark at room temperature for 1 hr
8. Read on spectrophotometer at 560 nm with the lid removed

Research Operating Procedure 11: Mincing of Liver Samples

Small Scale

1. Measure the weight of a sterile 50ml conical flask
2. Place approximately 1g liver tissue dissected from at least 3 independent liver samples into a sterile petri dish in a biohazard cabinet.
3. Slice each of the liver samples as thinly as possible using a scalpel blade
4. Dice the liver sample into small cubes (approximately $1mm^3$) and transfer to the sterile conical flask using sterile forceps.
5. Measure the weight of the conical flask and calculate the exact weight of the liver sample
6. Add 10ml of RPMI to the liver samples and store overnight at 4^oC.

Large Scale

1. Measure the weight of a sterile 200ml conical flask
2. Set up the mincer by placing the blade and plate with the larger holes into the mincer
3. Place approximately 10g liver tissue dissected from at least 3 independent liver samples into a sterile petri dish in a biohazard cabinet.
4. Place the liver tissue into the mincer
5. Turn the mincer on and collect the minced liver tissue in a sterile petri dish
6. Transfer the mincer liver tissue into the conical flask using sterile forceps
7. Measure the weight of the conical flask and calculate the exact weight of the liver sample
8. Add 30ml of RPMI to the liver samples and store overnight at 4^oC.

RPMI

RPMI-1640 Powder 10.4g/L (Thermo 50-020-PC)
Sodium Bicarbonate Solution 17.8ml/L (Pfizer DW99)
Penicillin / Streptomycin 10ml/L (Thermo SV30010)
Adjust pH to 7.2

Research Operating Procedure 12: Disaggregation of Liver Tissue

1. Preheat Shaking incubator and 0.05% collagenase (Invitrogen 17101015) solution to 37^{O}C.
2. Remove liver samples from 4^{O}C and transfer to a sterile biohazard cabinet.
3. For small scale disaggregation, remove the medium with a transfer pipette
4. For large scale, pass the liver and medium mixture through a metal strainer and transfer the tissue to the conical flask with a sterile metal spoon
5. To each flask add 0.05% collagenase solution (small scale: 6ml per 5g liver and large scale: 30ml per 30g liver tissue)
6. Transfer conical flasks to the shaking incubator and shake at 37^{O}C for 30 mins
7. Transfer the flasks to the biohazard cabinet
8. Small Scale: Collect the cells in the disaggregation solution using a plastic transfer pipette. Transfer the cells to a 10ml centrifuge tube
9. Large Scale: Pass the liver tissue and disaggregation solution through a metal strainer and collect the cells into a clean, sterile beaker. Transfer the liver tissue back to the conical flask using a metal spoon. Transfer the cells into a 10ml centrifuge tube
10. Collect the cells by pelleting at 800rpm for 10 mins in a benchtop centrifuge
11. Remove the supernatant and resuspend the cells in 10ml HMM containing 20% FCS.
12. Incubate the cells on ice until the disaggregation process is complete
13. Add fresh 0.05% collagenase solution to the liver tissue as per step 5.
14. Repeat steps 6 to 12 for another 3 collections (total of 4 collections, or 2 hours)

HMM (Hepatocyte Minimal Medium)

MEM – Eagle 6.27 g/L (Sigma M3024)
Medium 199 – 2.14g/L (Thermo 50-049-PA)
Sodium Pyruvate – 2.29 g/L (Sigma P5280)
L-Glutamine – 0.58g/L (Sigma G8540)
Sodium Bicarbonate solution – 17.8ml/L (Pfizer DW99)
Bovine Serum Albumin – 1 g/L (Sigma A1470)
Penicillin / Streptomycin – 10ml/L (Thermo SV30010)
Adjust pH to 7.2

Research Operating Procedure 13: Isolation of Liver Cells

1. Liver cells are disaggregated from liver tissue as described in Research Operating Procedure 12: Disaggregation of liver tissue.
2. Pellet the cells at 800rpm for 10 mins in a benchtop centrifuge
3. Remove the supernatant and resuspend the cells in 10ml serum-free HMM
4. Pellet the liver cells by centrifugation at 50 x g resulting in the red blood cells staying in the supernatant
5. Remove the supernatant and resuspend cells in 10ml serum-free HMM
6. Repeat steps 4 and 5 for a total of two centrifugations at 50 x g.
7. To remove any liver tissue chunks that remain in the sample, set up the following:
    a. A sterile beaker (sterile)
    b. Small metal strainer (sterile)
    c. Cell filter mesh (sterile)
8. Gently pass the cell suspension through the cell filter mesh and collect the cells into the beaker below
9. Transfer the cells into a centrifuge tube
10. Count the number and viability of the cells following Research Operating Procedure 5: Cell and viability counts using a haemocytometer.

HMM (Hepatocyte Minimal Medium)

MEM – Eagle 6.27 g/L (Sigma M3024)
Medium 199 – 2.14g/L (Thermo 50-049-PA)
Sodium Pyruvate – 2.29 g/L (Sigma P5280) L-
Glutamine – 0.58g/L (Sigma G8540)
Sodium Bicarbonate solution – 17.8ml/L (Pfizer DW99)
Bovine Serum Albumin – 1 g/L (Sigma A1470)
Penicillin / Streptomycin – 10ml/L (Thermo SV30010)
Adjust pH to 7.2

Research Operating Procedure 14: Liver Cell Plating

1. Liver cells are to be plated into serum-free HMM
2. To seed cells into 96 well trays, cells are plated at densities from 10,000 to 20,000 cells per well in 200µl of HMM
3. To seed cells into 6 well trays, plate 2.5×10^6 cells per well into 1.5ml HMM
4. To seed T25 flasks, plate 6.51×10^6 cells per flask added to 5ml HMM
5. To seed T75 flasks, plate 1.56×10^7 cells per flask added to 12ml HMM
6. To seed T175 flasks, plate 3.65×10^7 cells per flask added to 40ml HMM
7. Incubate cells in a CO_2 incubator at 37^oC and 5%CO_2 overnight to allow cells to adhere to the tissue culture dish or flask

HMM (Hepatocyte Minimal Medium)

MEM – Eagle 6.27 g/L (Sigma M3024)
Medium 199 – 2.14g/L (Thermo 50-049-PA)
Sodium Pyruvate – 2.29 g/L (Sigma P5280)
L-Glutamine – 0.58g/L (Sigma G8540)
Sodium Bicarbonate solution – 17.8ml/L (Pfizer DW99)
Bovine Serum Albumin – 1 g/L (Sigma A1470)
Penicillin / Streptomycin – 10ml/L (Thermo SV30010)
Adjust pH to 7.2

Research Operating Procedure 15: Stimulation of Liver Cells

1. Prepare the stimulatory factors by dissolving them in the appropriate solvent to an appropriate concentration (See Table 1).
2. Calculate the volume of medium required for each treatment and prepare a mastermix of each stimulant at the desired concentration in serum-free HMM.
3. Concentrations of each stimulant should be within the range summarised in Table 1.
4. Remove liver cells from the incubator that have adhered overnight following Research Operating Procedure 14: Liver Cell Plating.
5. Label the wells or flasks with the concentration and stimulant added
6. Aspirate the medium from the wells or flasks
7. Carefully add the medium containing the correct concentration and stimulant to each well / flask
8. Return the trays or flasks to the incubator and incubate the cells at 37^{O}C and 5%CO_2 for 48 hours.

Collect the medium and transfer to a freshly labelled 96 well tray or 10ml centrifuge tubes and store at -20^{O}C until ready to assay.

Stimulant	Solvent	Stock Conc	Minimum Stim Conc	Maximum Stim Conc	Company	Product Number
Calcium Ionophore	DMSO	1mM	10^{-9}M	10^{-5}M	Sigma	C9400
Retinoic Acid	DMSO	0.1M	10^{-8}M	10^{-6}M	Sigma	R2625
Phorbol Ester (PMA)	DMSO	1mM	50nM	200nM	Sigma	P148
Arachidonic Acid	Ethanol	100mM	0.5µM	5µM	Sigma	A3555
Desferrioxamine Mesylate Salt	Water	500mM	100µM	500µM	Sigma	D9533
Cobalt Chloride	Water	0.5M	50µM	500µM	Sigma	15862
Insulin	Acidified water	10mM	250nM	1000nM	Sigma	I6634
Glucose	Water	1M	10mM	50mM	Sigma	G7528
Dexamethasone	Methanol	100mM	1nM	100nM	Sigma	D6645

Table 1. List of liver stimulants with the solvent used, stock concentration and range of concentration used for stimulating bioactive production. The ordering information is also provided

HMM (Hepatocyte Minimal Medium)

MEM – Eagle 6.27 g/L (Sigma M3024)
Medium 199 – 2.14g/L (Thermo 50-049-PA)
Sodium Pyruvate – 2.29 g/L (Sigma P5280)
L-Glutamine – 0.58g/L (Sigma G8540)
Sodium Bicarbonate solution – 17.8ml/L (Pfizer DW99)
Bovine Serum Albumin – 1 g/L (Sigma A1470)
Penicillin / Streptomycin – 10ml/L (Thermo SV30010)
Adjust pH to 7.2

Research Operating Procedure 16: basic Fibroblast Growth Factor ELISA

1. R&D Systems ELISA Kit (DY233)
2. Reconstitute **Capture Antibody** in 1ml PBS (results in a concentration of antibody at 360µg/ml)
3. Aliquot **Capture Antibody** and store at -20^{O}C
4. Reconstitute **Detection Antibody** in 1ml Reagent Diluent (1% BSA in PBS, filter sterilised) (results in a concentration of antibody of 45µg/ml)
5. Aliquot **Detection Antibody** and store at -20^{O}C
6. Reconstitute 1 vial of FGF-2 Standard with 0.5ml of Reagent Diluent (results in a concentration of standard of 80ng/ml).
7. Allow the standard to sit for 15 minutes with gentle agitation prior to making dilutions.
8. Store reconstituted standard at -70^{O}C.

Plate Preparation

1. Dilute the capture antibody to the working concentration (2µg/ml) in PBS without carrier protein.
2. Immediately coat the 96 well microplate with 100µl per well.
3. Seal the plate and incubate at room temperature overnight
4. **The Next Day:** aspirate each well and wash with wash buffer (0.05% Tween 20 in PBS).
5. Repeat wash 2 times for a total of 3 washes
6. Blot paper on clean towels to remove any remaining wash buffer
7. Block plates by adding 300µl of reagent diluent to each well
8. Incubate at room temperature for at least 1 hour
9. Repeat aspiration and wash step as per steps 4 and 5.
10. Plates are ready for addition of samples

Assay Procedure

1. Add 100µl of sample or standards in reagent diluent per well (Standards should consist of 2-fold dilutions of stock standard, utilising 7 points of a standard curve with a high standard of 1000pg/ml)
2. Cover plate with adhesive strip and incubate for 2 hours at room temperature
3. Repeat the aspiration and wash steps as per steps 4 and 5 above
4. Dilute detection antibody to a working concentration of 0.25µg/ml in reagent diluent and add 100µl of detection antibody to each well
5. Cover with a new adhesive strip and incubate at room temperature for 2 hours
6. Repeat the aspiration and wash steps as stated in steps 4 and 5 above
7. Add 100µl of the working dilution of Streptavidin-HRP to each well (as stated on the bottle) to each well. Cover the plate and incubate at room temperature for 20 minutes in the dark
8. Repeat the aspiration and wash steps as stated above in steps 4 and 5
9. Add 100µl of substrate solution (1:1 mix of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) to each well and incubate for 20 minutes at room temperature in the dark
10. Add 50µl of Stop Solution (2 Molar Sulphuric Acid) to each well. Gently tap the plate to ensure thorough mixing

11. Determine the OD of each well immediately using a microplate reader set to 450nm with wavelength correction set to 540 or 570nm.
12. Calculate results by plotting standard curve

Research Operating Procedure 17: Platelet Derived Growth Factor ELISA

1. R&D Systems ELISA kit (DY220)
2. Reconstitute vial of PDGF Standard with 0.5ml of Reagent Diluent (results in a concentration of standard of 130ng/ml). Mix thoroughly.
3. Wash Buffer: to make 1 litre, add 50ml of 20x PBS to 950 ml RO Water. Add 500µl of Tween20 (Level 5 chemical store).

Plate Preparation

4. Dilute the capture antibody (abcam (ab38561); stored at $4^{O}C$) to 1/5000 (i.e. 1µl of antibody per 5ml PBS) in PBS without carrier protein.
5. Immediately coat the 96 well microplate with 100µl per well.
6. Seal the plate and incubate at room temperature overnight

7. **The Next Day:** Remove capture antibody and wash plate 3 times in wash buffer (0.05% Tween 20 in PBS).
8. Blot plate on clean absorbent paper to remove any remaining wash buffer
9. Block plates by adding 300µl of reagent diluent to each well
10. Incubate at room temperature for at least 1 hour
11. Remove reagent diluent and wash plates 3 times with wash buffer.
12. Plates are ready for addition of samples

Assay Procedure

13. Add 100µl of sample or standards in reagent diluent per well (Standards should consist of 2-fold dilutions of stock standard, utilising 7 points of a standard curve with a high standard of 2000pg/ml)
14. Cover plate with adhesive strip and incubate for 2 hours at room temperature
15. Remove standards and samples and wash plate 3x with wash buffer
16. Dilute detection antibody to a working concentration of 400ng/ml in reagent diluent (stock at 72µg/ml) and add 100µl of detection antibody to each well
17. Cover with a new adhesive strip and incubate at room temperature for 2 hours
18. Remove detection antibody and wash plate 3x with wash buffer
19. Add 100µl of the working dilution of Streptavidin-HRP to each well (1/200 dilution) to each well. Cover the plate and incubate at room temperature for 20 minutes in the dark
20. Remove streptavidin-HRP and wash plate 3x with wash buffer
21. Add 100µl of substrate solution (1:1 mix of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) to each well and incubate for 20 minutes at room temperature in the dark
22. Add 50µl of Stop Solution (2 Molar Sulphuric Acid) to each well. Gently tap the plate to ensure thorough mixing
23. Determine the OD of each well immediately using a microplate reader set to 450nm with wavelength correction set to 540 or 570nm.
24. Calculate results by plotting standard curve

9.2 Abbreviations

2-D	2-dimensional
^{o}C	Degrees centigrade
ACN	Acetonitrile
cDNA	Complementary deoxyribose nucleic acid
cG	Chorionic gonadotrophin
CL	Corpus luteum
cm^2	Centimetres squared
$CoCl_2$	Cobalt Chloride
Coll	Collagenase
db-cAMP	Dibutyryl cyclic adenosine monophosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked Immuno-sorbent Assay
Epo	Erythropoietin
FGF2	Fibroblast Growth Factor -2
FCS	Foetal Calf Serum
g/l	Grams per litre
g	Grams
HBSS	Hank's Buffered Saline solution
HMM	Hepatocyte Minimal Medium
HPLC	High Pressure Liquid Chromatography
IGF2	Insulin-like Growth Factor-2
kDA	Kilo-dalton
LC	Liquid chromatography
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionisation – Time of Flight – Mass Spectrometry
mg	Milligrams
mm	Millimetres
MS	Mass Spectrometry
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
m/z	Mass-to-charge ratio
ng/ml	Nanograms per millilitre
nM	Nanomolar
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
pg/ml	Picograms per millilitre
PMA	Phorbol 12-myristate 13-acetate
RA	Retinoic Acid
RIA	Radioimmunoassay
RNA	Ribo-Nucleic Acid
RT-PCR	Reverse Transcription – Polymerase Chain Reaction
RT	Reverse Transcription
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis

SPE	Solid Phase Extraction
TE	Trypsin-EDTA
TNF-alpha	Tumour Necrosis Factor - alpha
ug/ml	Micrograms per millilitre
ul	Microlitres
U/ml	Units/millilitre