Investigating Potential Biochemical Properties of Fetal Membrane Spongy Layer for Clinical Application at the Ocular Surface

Elena Alexandrovna Lazutina, MD

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Fairy tales are more than true: not because they tell us that dragons exist, but because they tell us that dragons can be beaten.

— G.K. Chesterson (from Coraline by Neil Gaiman)

Abstract

The amniotic membrane, well known scaffolding tissue, which widely uses and benefits of having anti-inflammatory, anti-microbial, anti-fibrosis, anti-scarring with low immunogenicity and reasonable mechanical properties.

Amniotic Membrane Transplantation (AMT) is an established treatment modality, which favourably influence ocular surface re-epithelisation and prevents angiogenesis, thus promoting healing and minimising scarring. It is also used at several other sites of the body. Despite its widespread use, key elements of the membrane and its precise mechanism(s) of action remain to be elucidated. Unfortunately, over the years conflicting clinical reports have suggested variations in the efficacy of AM utility. Conventional methods for amnion preparation do not acknowledge the presence of the SL.

My project is a continuation of previous PhD completed in the department, which mentioned the Spongy Layer as one of the important layers in Amniotic Membrane, which had not been look for in any previous work and Dr A Hopkinson, the author of previously mentioned PhD, accidentally discovered possibility to separate that layer from the amnion, it had been decided to take a close look at the layer and investigate properties.

Researchers at the University of Nottingham have developed and improved techniques of manufacturing clinical grade the amnion and they have identified the SL as a substance that is variably present in conventional amnion. They have developed techniques to entirely isolate the SL, which allows comprehensive characterisation of its composition and biological properties.

The project originally designed to investigate all layers of Amniotic Membrane separately in comparison with amniotic membrane which is completely free from Spongy Layer (SL detached) as well as Spongy Layer attached to the amniotic membrane (classically used layer) and identify a layer which is richest in proteins and growth factors. So, three different samples were investigated:

1) Isolated Spongy Layer;

2) Isolated Amniotic Membrane;

3) Amniotic Membrane with Spongy Layer attached (classical layer, which well-known used).

I used technique developed in the department. This technique allows separating the SL without unnecessary mechanical tissue disturbance and isolated SL was used to investigate comprehensive characterisation of the composition and biological properties.

Our technique of removing SL is simple and could be easily adapted. The SL imbibes water well and significantly expands, which makes it thick and easy to pull using forceps or using blunt edge of the scalpel blade to push the SL from the amnion surface without mechanical interruption.

Investigation of origin of the Spongy Layer during gestation period done through intensive literature search and came to conclusion that the Spongy Layer developed from the extraembryonic endoderm. It is well known that the SL acts as a barrier between vascular amnion and avascular chorion.

Also, question was about similarities and differences of embryological origin of the Wharton Jelly and the Spongy Layer. The present in which of TGFb1 (immunofluorescent staining) could be result of cross link during the embryological development.

As previously, reported by Dr A Hopkinson et al in 2006, that the Spongy Layer's biochemical composition is containing TGF-b1, EGF and HGF. The structure of SL is reported to be composed of Collagen types I-VIII, the SL contains high level of hyaluronan, which is a major carbohydrate component of the ECM.

To extract proteins from the SL, a few different techniques were used, first of all the tissue weighted, freeze dried and lyophilised in buffers, which were different and depended on the experiment planned.

After, proteins were analysed through the Searchlight protein array analysis, 2-D protein quantitation, the Bradford (Commassie staining) assay, the mass spectrometry, had been discovered that the SL contains angiogenic factors,

biomarkers, cell adhesions factors, cytokine proteins, growth factors, metalloproteinases, chemokine proteins, neurotrophic factors and cellular components. Experiments show that the level of those factors and proteins in fresh AM, the Transplant Ready Amniotic Membrane (TRAM) and the SL shows that significant amount of proteins was simply washed out during preparation from the TRAM, however the SL is holding those proteins in significant level probably due to imbibing while absorbing water.

The important discovery of this project was the cytotoxic effect of the SL and its antimicrobial properties.

Some fractions of the SL with high molecular weight proteins show apoptotic activity to corneal keratofibroblasts by necrosis rather than apoptosis. However, cells occurred apoptosis after treatment with lower molecular weight proteins. As preliminary data shows the SL to be cytotoxic, this could lead to some understanding of different outcome in the Amniotic Membrane transplantation (AMT). However, this area needs further investigation.

Well known antimicrobial properties of the amniotic membrane were established only in the TRAM and in the samples of the SL which were prepared in the same way as TRAM (washed in gentamicin), however samples of the SL which were not washed in gentamicin did not show antimicrobial properties.

One of the next steps of investigation properties of Spongy Layer was the measurement of the thickness of the layer in normal physiological situation, during gestation. As the SL is imbibing the water quickly, in vitro it was difficult to measure “real” thickness of the membrane; this gave an idea to measure it in vivo. To answer the question of “real” (physiological) thickness of the Spongy Layer, different methods were used, however the Ultrasound technique was our method of interest, as it gave the possibility to see the layer during gestation without any interruption and see changes in thickness prior to delivery. These measurements were done in the Fetal Maternal Medicine Department by very experienced sonographer. The Spongy Layer has a variable thickness in three

different areas (cervical part, mid region and apical part of the uterus) the SL regions had been divided accordingly.

The Spongy Layer has a variable thickness depending on the anatomical location. The difference of the SL thickness had been measured in vivo (ultrasound technic – described below) and in vitro (this work published by JJ Gicquel) the compared results matched.

The major problem of the project was the separation of proteins and to break hyaluronic chains to extract clean proteins.

In my project, I used two different techniques to separate proteins according the molecular weight of proteins presented in the sample.

1) Revers Phase Solid Phase Extraction (RP-SPE) isolation of proteins; and
2) Soluble Protein Fractionation using Vivaspin columns

The second technique in my hands was more successful and I decided to use this for all my samples. To minimise the possibility of sample variations from the point of sample preparation of the SL and the AM itself, I used the same technique for all experiments and combined samples.

Nevertheless, my samples were not 100% clear due to different protein structure and shape.

This layer therefore has the potential to be exploited clinically for the treatment of several indications. However, before it can be employed, the layer requires further investigation to determine characterise the content of potential factors. In addition, as the spongy layer is predominantly composed of mucin and proteoglycans resulting in a gelatinous/viscous substance, a processing procedure must be developed to either modify the substance in a usable format, or to extract the beneficial factors, for clinical use.

Being able to demonstrate the isolated SL and its derivatives can be exploited as potential therapeutic agents in the treatment of many ocular surface disorders, would have significant translational potential. The control of inflammation caused

by disease (e.g. Ocular cicatricial pemphigoid) and any injury (e.g. Chemical burns) and the limitation of scarring and vascularisation would preserve sight or allow successful secondary intervention such as corneal drafting, which otherwise has a high risk of failure in such situations, the potential for preventing visual impairment and promoting quality of life in all age groups in therefore immense.

My work proved that the SL is a separate layer and is having a vast number of different factors in a significantly high amount compare to amnion itself.

Acknowledgements

Firstly, I would like to thank you Professor Harminder S Dua, who agreed to take me on as one of his research students.

Thank you to Dr Andrew Hopkinson for full project design, constant support in all situations, his availability to discuss any issue arrived during the project at any time. And his huge number of patients, understanding, especially, when something was going wrong and teaching me with clinical background, of all laboratory rules.

The fundamental requirement for this research was the unrestrained availability of foetal membranes, which would not have been possible without the collaboration of Professor DK James and the department of foetal-maternal medicine for collaboration in collection membrane and providing ultrasound measurement of the thickness of the amniotic membrane.

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Also, thank you to my other fellow PhD students in the past who shared all laboratory work experience (which was new, exciting and often not easy to understand for people with clinical background), Dr Aaron Yeung, Dr Naing Lat Tint, Dr Gerry Claire, Dr Khurram Hashmani. Dr Elizabeth Stewart, who thought

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List of abbreviations

AECs – amniotic epithelial cells

AF – amniotic fluid

AM – amniotic membrane

AMT – amniotic membrane transplantation

And2 – angioprotein 2

BDNF – brain derived neurotrophic factor

BM – basement membrane

CEC – corneal epithelial cells

CNTF – ciliary neurotrophic factor

DMEM – Dulbecco's Modified Eagle's Medium

ECM – extracellular matrix

EGF – epidermal growth factor

ELISA - enzyme-linked immunosorbent assay

EM – electron microscopy

ENT – ear nose and throat

FASL – fas ligand fibrinogen

FBS - fetal bovine serum

FGF – Fibroblasts Growth Factor

GDNF – glial cell line-derived neurotrophic factor

H_2O – water

HAEC – human amniotic epithelial cells

HBEGF – heparin-binding growth factor

HGF – hepatocyte Growth Factor

HGH – human growth hormone

HIV – human immunodeficiency virus

HTA – human tissue authority

ICAM – intercellular adhesion molecule

IFN – interferon

IL – interleukin

KGF – keratocyte Growth Factor

LN2 – liquid nitrogen

LSCD – limbal stem cells deficiency

MHRA – medicine and healthcare products regulatory agency

MIF – migration inhibitor factor

MIP – macrophage inflammatory proteins

MMP – matrix metalloprotease

MSC – mesenchymal stem cells

NaCl – sodium chloride

NGF – nerve growth factor

NHS - National Health Service

NT – neurotrophin

PARK – photoastigmatic reflective keratectomy

PCR – polymerase chain reaction

PEDF – pigment epithelium derived factor

PROM – premature rupture of the membrane

RANTES – regulated on activation normal T cells expressed and secreted

RNA – ribonucleic acid

SC – stem cells

SCF – stem cells factor

SL – spongy layer

TBSTx – tris-buffered saline and tween 20

TGF – transforming growth factor ß

TIMP - tissue inhibitor of metalloproteinase

TNF – tumor necrosis factor

TRAIL –-related apoptosis-inducing ligand

TRAM – transplant ready amniotic membrane

TSP – thrombospondin

VCAM – vascular cells adhesion protein

VEGF – vascular endothelial growth factor

WJ - Wharton jelly

UV - Ultraviolet-visible

Chapter 1 – Introduction: General introduction, anatomy of Amniotic membrane, and historical background of using AM in medicine and in ophthalmology. SL as a part of AM.

1.1 Amniotic Membrane (AM)

AM is component of the extraembryonic membrane, and has a long history of use in surgical applications, including gynaecology [1-3] traumatology [4, 5] dermatology [6], dental surgery [7] ear, nose and throat surgery [8], orthopaedics [9] and ophthalmology [10-12]. Initial research using AM for treatment of skin burns [13] and in bladder reconstruction [14] and plastics surgery [15] portrayed AM as a potential “miracle” therapy. Since these initial findings there have been great strides in researching AM, to elucidate its unique properties. In more recent years AM has been shown to have anti-inflammatory [16], anti-angiogenic [17], anti-microbial [18] and tumorigenic [19] properties, and is suggested to be a possible source/niche of Mesenchymal Stem Cells (MSCs) [20].

AM has been most successful in its use for ocular surface reconstruction [21-23]. However, recent emerging data suggests that variation in biochemical composition between donors may be the cause of less favourable clinical efficacy [24, 25]. The extraembryonic coelom or spongy layer (SL) is the gelatinous, protein- rich layer, on the stromal aspect of the AM, which develops between the amnion and the chorion, collectively known as a foetal membrane [26]. This SL often remains associated with the AM and is routinely overlooked during clinical processing of AM for transplantation, therefore as a result it is partially but variably removed during preparation by mechanical rubbing. The foetal membrane is are composed of many components and proteins such as thrombospondin, mimecan, TGFB1 (Transforming Growth Factor, beta 1 initially called BIG-H3) and integrin alpha 6 [27], some of proteoglycan rich components, predominantly smaller such as biglycan [28] and decorin [29, 30].

1.2 Amniotic Membrane structure

AM is an avascular transparent structure that forms the innermost lining of the fetal membrane that enclose the developing fetus (Figure 1). Human AM is composed of five distinct layers (epithelium layer, basement layer, compact layer, fibroblast layer and spongy layer) (Figure 1 Fetal membrane structure by Bourne et al 1960.).

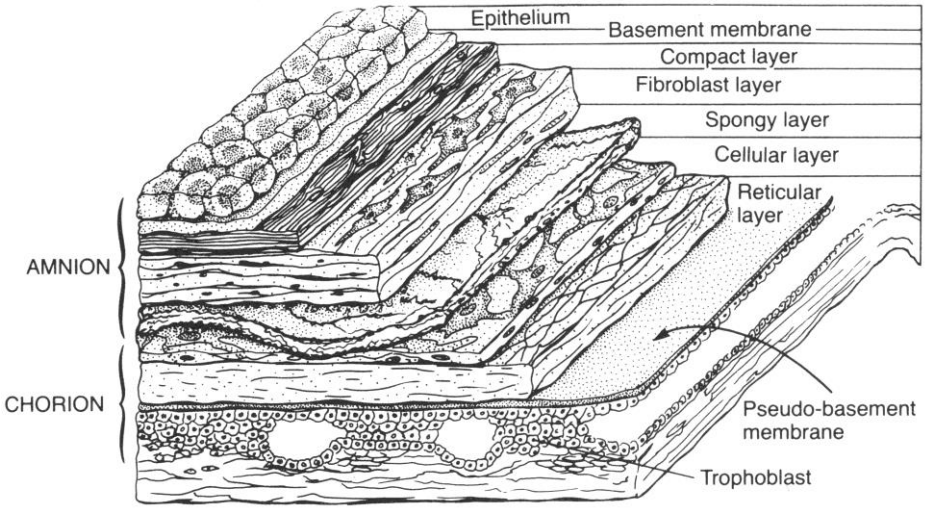


Figure 1 Fetal membrane structure by Bourne et al 1960.
A diagram of fetal membrane structure(taken from Bourne et al 1960[31]). The diagram shows the different layers of the foetal membrane including a highly organised epithelial layer, a collagen dense compact layer. Amnion contains four layers: epithelium, basement membrane, compact layer and fibroblast layer: chorion four layers; cellular layer, reticular layer, pseudo-basement membrane and trophoblast. The spongy layer is in the middle of the foetal membrane; however, Bourne described the spongy layer as a fifth layer of the amnion.

1.2.1 Epithelial layer

A single layer of epithelial cells (thickness 15+/- 1μm) form the innermost layer of AM. These cells are surmounted by many tiny basal processes, which protrude into the basement membrane (BM). A complex intercellular system is present consisting of branching canals and may partly include the wall of the cells. Microvilli also protrude into these canals. The complex system of tiny intracellular canals, which connect between the intercellular canalicular system and the base of the cell can be observed using high magnification electron microscopy (EM) [31].

It is suggested that, there are two types of amniotic epithelial cells (AECs) [32, 33] [34-37]. A single layer of cuboidal cells with a basal surface supported by a

dense acellular collagenous matrix, form the amnion reflectum (amnion forming the sac) [38]. Irregular, branched and confluent cells cover the apical surface, which continue down lateral boarders of the cuboidal cells to form intercellular canaliculi or channels of labyrinthine type [39]. Continuous cells are joined with lateral desmosome-like complexes, but no tight junctions are formed [38].

The second type of epithelial cells line the placental AM (the amnion attached to the placenta itself). They are morphologically different to AECs of the reflectum [40]. They are cylindrical and more stratified, with more pronounced perinuclear vacuoles, indicating increased secretion potential. The nuclei are located apically in cells that are more columnar. Increased production of cytokeratins 1, 10 and 11 make these cells appear more stratified [41].

AECs predominantly secrete collagen types I, III [35] and collagen type IV via the BM and non-collagenous glycoproteins e.g. laminin, nitrogen and fibronectin, that form the BM. Other researchers shows, one layer of epithelial cells in the amnion. [38, 42]

1.2.2 Extracellular matrix

The ECM can be considered an architectural supportive framework, providing tensile strength to the membrane, maintaining mechanical integrity and the fluid environment. The ECM is a collective of the BM and compact layer. The primary constituents of this sub-layer are fibrous proteins that assemble to form a meshwork, embedded with polysaccharides [43]. Collagen types I and III are fibrillar components of the interstitial collagens and have a complimentary supportive role in tissue strength and function.

1.2.2.1 Basement membrane

The BM is a narrow layer of compact connective tissue consisting of a complicated reticular network, responsible for AM strength. The BM consists of a lamina-densa and a lamina-rara (lucida). At the distal tips of basal cells, the processes at the lamina-rara are narrow (30 nm) [38], with frequent small

parallel fibrils running from the lamina-densa to the cell surface. Laminin is a major component of the BM and is formed from several subunits, linked by disulphide bonds. Like fibronectin, laminin has multiple cell attachment sites, but these sites differ from that of fibronectin. Laminins are classified as products of AECs [38]. Laminin-7 is characterized as a novel amniotic laminin, associated with laminin-5 [44]. Laminins are involved in anchoring AECs and BM to the underlying compact layer, via collagen type VI.

1.2.2.2 Compact layer

The compact layer (thickness 30+/-4μm) consists of a complicated reticular network that is acellular and comprised of a dense network of parallel undulating bundles of fibres [35, 38], one of the main collagen fibril containing layers of the fetal membrane [45]. It is a dense fibrillar transitional zone, visible between the lamina densa and the stroma. This traditional zone contains predominantly 18 nm fibrils with occasional 25 nm fibrils present exclusively in this layer. Using transmission EM, Alpin *et al* showed the presence of amorphous “filler” material [38], possibly proteoglycan or other minor collagens. The compact layer plays a role in providing mechanical strength and maintaining the integrity of the fetal cavity during pregnancy. Type I and type III collagens are prevalent and are tightly packed throughout this layer [45]. The presence of collagen type IV and the fibril-associated collagen type XIV have also been demonstrated in AM [45].

AM has been described as visco-elastic, however histological and chemical efforts to identify components within the ECM associated with elasticity have been difficult. Elastin is an amorphous component of elastic fibres. These subunits are constructed outside the cell forming insoluble elastic fibres. The deposition and cross-linking of elastin is the final event to occur before the structure is formed on the fibrillin-based microfibrils [46]. The presence of fibrillin-based microfibrils in AM has been previously reported without the association of elastin [46]. These structures have also been demonstrated in the mesenchymal

and compact layers [47] with lack of elastin detection, suggesting that the elasticity was attributed to the microfibrils alone.

1.2.2.3 Fibroblast layer

The fibroblast layer (thickness 22+/-5μm) contains a loose network of fibroblasts and fibronectin with phagocytic functions. This layer is defined by bundles of fibres, with embedded fusiform and stellate-shape cells, in a less tightly fibre meshwork [39, 48]. Collagen type I and III are present in the fibroblast layer [45]. Extensive research in the last decade has eluded to the presence of Mesenchymal Stem Cells (MSC) in the fibroblast layer of the amnion [37, 49-51]. It been found, that fibroblast layer of the amnion contains also nitrogen, laminin, MSCs and CD14+monocytes[52].

1.2.2.4 Spongy layer (SL)

The SL is the inner most stromal layer of the AM composing of loose connective tissue. Distinguishing the SL ultra-structurally is difficult. When visualized, a gap between the fibroblast layer and the reticular layer is a pattern of complementary gyri (peaks) and sulci (cleft), implying an ability to combine tensile strengths of both layers by interlocking. The AM and chorion differ in radii and curvature. Stresses triggered by changes in AF volume or fetal movement are overcome by mutual sliding of the AM over the chorion. It is postulated that the SL plays a role in this movement enabling AM to move comparatively freely upon the fixed underlying chorion.

1.3 Use of Amniotic membrane

1.3.1 History of amniotic membrane uses in clinics.

The first clinical application of AM, outside its physiological role was in 1910. The amnion was used as a biological wound dressing in the treatment of severe burns to the skin [53], chemical and thermal. Subsequently in 1913, Sabella reported successful use of AM in the treatment of skin ulcerations [54, 55]. Thereafter it has been used in surgical procedures related to other systems within the body [56-58] such as gynaecological, abdomen, urological and shows successful usage of the AM in periodontal surgery [59].

Classically, AM transplantation (AMT) has developed through a non-ocular perspective. This tissue has been demonstrated by its use in the surgical reconstruction of the abdominal cavity [10, 58, 60, 61] vagina [57, 62] urethra and the oral cavity [63, 64] and as wound dressings [65] in dermatology [66].

1.3.2 Amniotic membrane in ophthalmology.

The first documented ophthalmological application was in the 1940s, in the treatment of ocular burns [12, 55, 58, 67]. Following initial reports, its use in ocular surgery abated until recently when its potential was re-discovered in the Soviet Union and South America. Its introduction to North America in the early 1990s heralded a vast surge in ophthalmic applications [55, 58, 62, 68-71].

Ophthalmic indications encompass ocular surface reconstruction in stem cells (SC) deficiency; treatment of persistent epithelial defects [72-74]; conjunctival reconstruction following surgery for symblepharon, pterygium, and excision of lesions such as tumours, granulomas and bullous keratopathy [55, 75-88]. AM use is also recommended in glaucoma surgery for the treatment of leaking blebs, scarring, and as an overlay to tube shunts [58, 78, 89-93]. In oculoplastic surgery AM has been successfully used in blepharoplasty and punctal occlusion. In addition AM may be used in photorefractive keratectomy to prevent haze, band keratopathy and keratoprosthesis [27, 87, 94-102].

AM can be used as a patch [55, 103], wherein it is applied as a ‘biological bandage’[62] to provide cover and protection; as a scaffold allowing expansion and growth of conjunctival or corneal epithelium; or as multiple layers [104] for tissue re-building. AM is applied usually epithelial side up favouring re-epithelialization or epithelial side down, facilitating the entrapment of inflammatory cells in the stroma, reducing inflammation [10, 12, 61, 105]. Two membranes may be used simultaneously, one epithelial side up and one epithelial side down, with the former acting as a graft (inlay technique, when AM secured attached with cornea defect and merged with host tissue [106]) (Figure 2) and the latter as a patch (overlay technique, AM used as a biological barrier [11, 107]) (Figure 3) to prevent conjunctival ‘contamination’ of limbal derived corneal epithelial cells (CEC) [108-110].

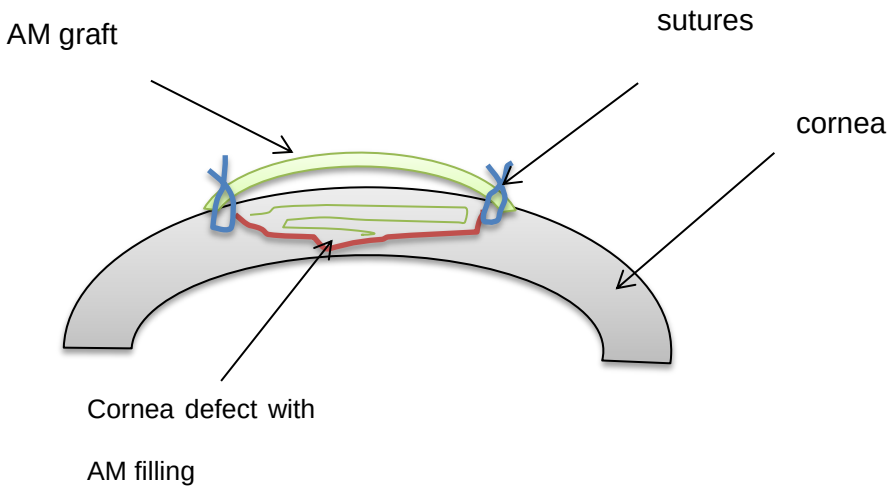


Figure 2 AM graft.
AM graft (schema). AM graft covers cornea defect with AM filling of the defect under the graft.

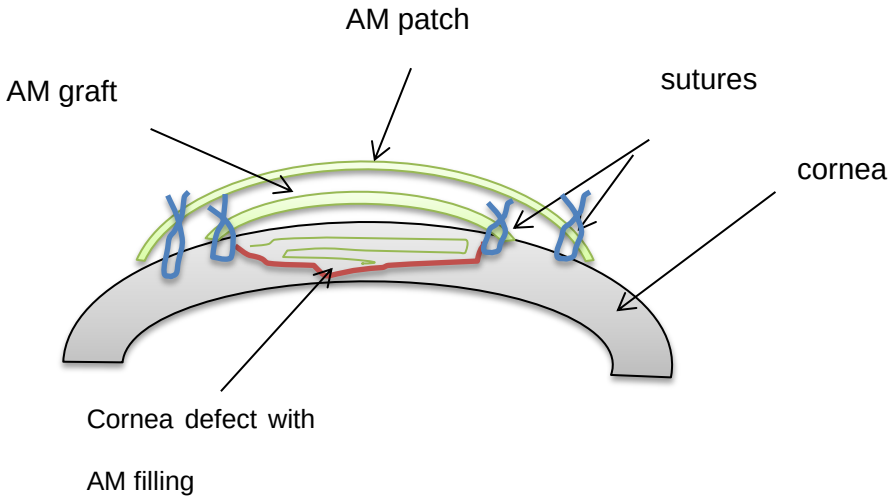


Figure 3 AM patch.
AM graft v patch (schema). AM patch used to cover wider area of the cornea defect on the top of coverage of AM filling and graft.

Surgeons in the Ophthalmology Department, University of Nottingham have been using the membrane for over 12 years and have published extensively within the scientific community [10, 12, 61, 111, 112]. It is evident, largely from our own published work, that the use of human AM for clinical indications is far from standardised. Several differences in cytokine and growth factor content have been shown to exist between membranes and between different sites of the same membrane [2, 111, 113].

AMT as a patch is usually combined with stem cell transplantation, and is performed simultaneously or subsequently after the surface has been adequately prepared by AMT [12, 114, 115]. This has been reported to reduce vascular in-growth, preserve corneal transparency and restore corneal-like epithelial phenotypes [116-120].

It has been documented that AM plays a functional role in the healing process of the ocular surface and provides a healthy and stable substrate for re-epithelialisation. Dua *et al* [12] suggested that when AM is sutured epithelial side down and matrix side up, the matrix traps inflammatory cells and induces apoptosis, thereby reducing inflammation [12, 61, 103, 104, 114, 121, 122].

Combining this with previous reports on AMT, one would assume that other biochemical factors [117, 123-125] and signalling pathways [126-130] are involved.

1.3.3 Ocular surface reconstruction

1.3.3.1 Cornea

1.3.3.1.1 Limbal stem cell deficiency

The ocular surface is structured from two types of epithelial cells, corneal and conjunctival epithelia. They continue into each other at the limbus. The limbus contains the SC, which act as a barrier between two cells type and prevent each other to migrate from one site to another. Under some pathological circumstances, for example Stevens-Johnson syndrome, chemical or thermal burns [131] and ocular cicarticial pemphigoid, SC may deplete causing conjunctival epithelial cell types to grow into the corneal wound site. This condition is known as “conjunctivalization” [11, 132-134] of the cornea, with vascularization.

Partial SC deficiency can be managed and AMT is a useful adjunct to the above procedures. Sangwan *et al* [135] described 60-70% success in patients treated with AM following pannus resection. However, in LSCD with partial conjunctivalisation of the cornea and mild vascularisation a beneficial outcome can be achieved with sequential sector conjunctival epitheliectomy, without using AM [136-144].

1.3.3.1.2 Pseudophakic bullous keratopathy

Using AMT to treat bullous keratopathy done by many ophthalmic surgeons, however Pires *et al* [79], achieved 91% of pain relief in patients in 33 weeks after treatment using AM. Following this finding, Espana *et al* [145] reported a pain free in 88% of patients in 25 month follow up[146]. Figure 4 showing an eye with bullous keratophaty, which had been treated in the Department of Ophthalmology

in Nottingham University Hospitals NHS Trust. In Figure 4 A, 10mm graft of the AM been sutured with nylon sutures, which were removed in 10 days after an operation. And Figure 4 B, showed the same eye 10 month later, bullous keratopathy treated successfully, however, AM became opalescent in the centre of the cornea.

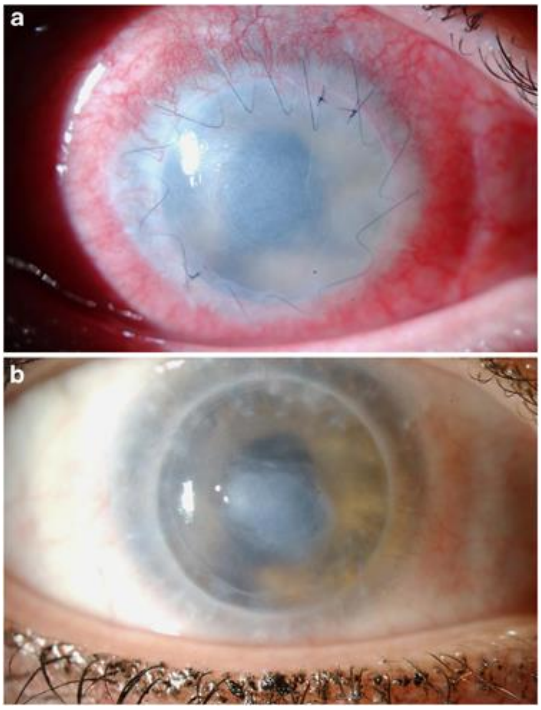


Figure 4 AM in treatment of bullous keratopathy.
The treatment of bullous keratopathy with AM. A) penetrating cornea graft treated with AM: B) the same eye 10 month later.

1.3.3.1.3 Persistent corneal epithelial defects and perforations

Persistent corneal epithelial defect can be with or without ulceration are a serious and urgent ophthalmic condition, which can be complicated by microbial infection [147]. Corneal epithelial defect and corneal perforations is a wide area where AM has been successfully used during last 20 years [148]. Tseng *et al [60]* showed that AM provides a new basement membrane, which is necessary for supporting adhesion and growth of epithelial progenitor cells including stem cells. Plus anti-inflammatory effect of the AM is valuable here. Azuro-Blanco *et al* [11] found, that the success of amniotic membrane transplantation (AMT) is in promoting epithelialisation of the stroma. Su and Lin et al [149] have successfully sealed corneal perforations using AM. Figure 5 illustrates usage of AM for persistent

epithelial defect treatment, done in The Department of Ophthalmology, Nottingham. On the Figure 5 A, membrane graft covered epithelial defect. The Figure 5 B, the same eye in eight-month post operatively, showed defect healed completely.

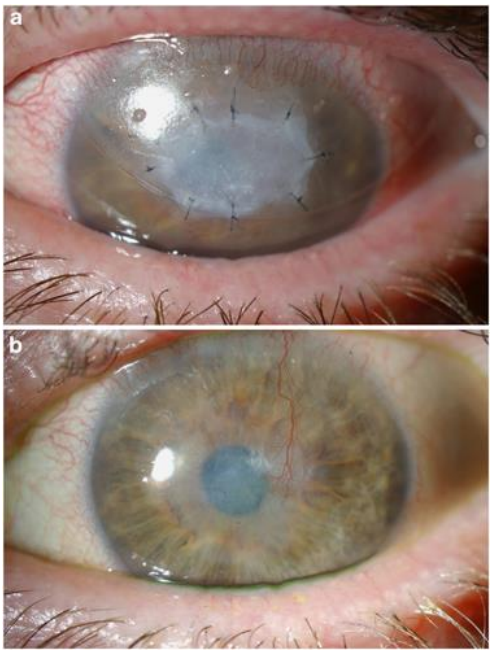


Figure 5 AMT in treatment of persistent epithelial defect.
AMT used to treat persistent epithelial defect. A) AM graft covering epithelial defect; B) the same eye eight month late, AM incorporated into cornea successfully, defect healed.

1.3.4 Conjunctiva

1.3.4.1 Pterygium

Pterygium can be treated using the different techniques. Recently, new approaches using fibrin tissue adhesive and amniotic membrane improved outcome of the surgery significantly[150]. Prabhasawant *et al* [23] first described the use of AM as an adjunct in the surgical treatment of pterygium, as an alternative to autologous conjunctival grafts. The head of pterygium was separated and dissected forward the central cornea with scissors, when head and most of the pterygium body were removed, AM graft was created and bare scleral area covered with AM basement side up. AM was sutured through the episcleral tissue to the edge of conjunctiva along the bare sclera border [151].

The relatively low number of patients with primary pterygium, been offered an AMT as a first choice of treatment.

1.3.4.2 Tumors

AM has been used in the treatment of both conjunctival and corneal tumours following surgical resection [152-154]. The conjunctival melanoma was completely excised, with wide clinically disease-free margins and AM immediately sutured to the surrounding conjunctiva and sclera to cover the conjunctival defect. In both studies AMT had a positive outcome with rapid healing of the defect and long-term ocular surface stability. However, in a second study some complications such as symblepharon formation and partial SC deficiency were reported. Figure 6 shows the eye diagnosed with conjunctival intraepithelial neoplasia, treated with AM in the department of ophthalmology ENT and Eye centre, Nottingham. Figure 6 A and Figure 6 B represent the same eye before treatment and after excision and AM cover in one week after surgery.

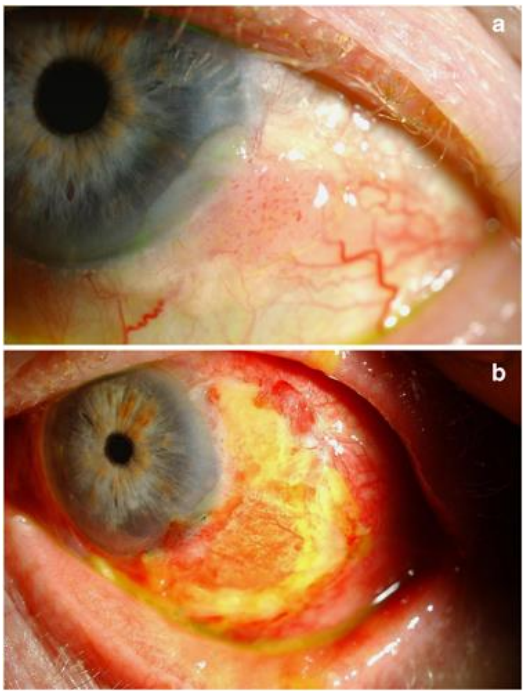


Figure 6 AM in treatment conjunctival intraepithelial neoplasia (CIN)
AM used to treat conjunctival intraepithelial neoplasia (CIN). A) conjunctival intraepithelial neoplasia; b) the same eye 1 week postoperatively, after excision and an AM cover.

1.3.4.3 Symblepharon

AM has been used for treatment and prevention of symblepharon as a patch. The main purpose of the patch is to use it as a bandage until full healing is achieved. AM was inserted into the superior fornix while the patient looked down and then slid under the lower eyelid. Generally, AM is replaced every 7-10 days. Solomon et al [91] and Barbabino et al [144] showed that AMT could be used in the treatment of symblepharon as a primary treatment option for ocular reconstruction. However, treatment effectiveness deteriorated over time in more than 50% of patients, as it can bring about pain reduction, symblepharon and entropion resolution, and end the continual damage to the ocular surface epithelia that results from recurrent erosions, ulcer formation and secondary bacterial infections[143]. Tseng [125] reported a good outcome in patients using a sutureless AM patch in acute alkali injuries. The outcome may depend on the time scale between injury and treatment.

1.3.4.4 Glaucoma

AM can be used in the treatment of glaucoma, for a few different purposes. These include the treatment of complications, mainly to reduce scarring and to act as a cover for valve procedures. Fugishima et al [155] used AM to prevent adhesion of the scleral flap to the overlying conjunctiva. In this study new surgical technique developed of using AM. Trabeculectomy performed with a limbal-based conjunctival flap using 0.4mg/ml of MMC (mitomycin-C) for 2 minutes and after the AM placed under the scleral flap and sutured. Additionally AM has been successfully used to cover exposed pericardial patches over the tube of Ahmed valves [80, 156], in the treatment of glaucoma.

1.3.5 Oculoplastics and orbit reconstruction

In 2005, Becerra et al [154] described the use of an AM allograft in conjunctival melanoma with eyelid involvement. Chen et al [157] have recently reported their

method of treating ocular pathology by isolating orbital adipose stem cells from patients, isolated and purified from AM [157]. Poonyathalang et al [158] used AM grafts in forniceal reconstruction with a high success rate and limited complications. Finger et al [159] used AM to protect the cornea from radioactive eye plaques, with no significant effect on radiation dose. Results showed that AM was effective in reducing pain and protecting the cornea [160].

1.3.6 Drug reservoir

AM may also be used as a drug reservoir [161]. Resch et al [162] tested human amnion in vivo as a drug reservoir, they found that AM acted as an ofloxacin slow release device for up to 7 hours, depending on duration of pre-treatment of the AM. AM been soaked in the 3% ofloxacin ophthalmic solution and absorbance of the drug measured with Ultraviolet-visible (UV) spectrophotometer. Releasing amount of ofloxacin pro1cm^2 of AM (μg/cm^2) was calculated in the period of 1 to 90 min.

1.3.7 Astigmatism correction

The AM used to correct astigmatism, Zhou *et al* [163] performed photoastigmatic refractive keratectomy (PARK) on rabbits and grafted AM on the cornea. Measurement of degree of the astigmatism was by topography and cycloplegic refraction. Zhou *et al* [163] suggested that AM could be used for controlling the astigmatism induced by laser refractive surgery.

Strube et al [164] showed benefit on patients with restrictive strabismus caused by conjunctival scarring, fat adherence syndrome, or rectus muscle contractive. Authors performed AMT on patient's eyes, which developed restrictive strabismus after periocular surgery and treated with surgical removal of restrictive adhesions and placement of an AMT.

1.4 Mechanism of action of AM

1.4.1 Function of AM

The suggested mechanisms of action of AM include inhibition of scarring, inflammation, angiogenesis and the membrane is also thought to possess anti-microbial activity [165, 166]. In addition AM provides a substrate for epithelial cell growth and attachment both in vitro and in vivo for later transplantation onto the ocular surface. Many of these mechanisms of action are attributed to the presence of these beneficial molecules in 'fresh' membrane but our studies have shown that these may not be present in transplant ready amniotic membrane (TRAM) following processing and storage [27, 42, 72, 111, 117, 145, 167-170]. All described actions of AM and limitation of AMT are lead to biochemical compositions of the tissue. AM as classically used layer for the AMT include five layers (epithelial layer, basement membrane, compact layer, fibroblasts layer and spongy layer), four of the layers well know and vast worldwide research done to study those layers, however AMT variations brought to study last less known layer – spongy layer.

1.5 Limitations and side effects of AM

In the vast number of patients, AM has been a successfully used with reported successful rate of 43% with AMT against 3.4% of medical treatment [171]. As its mechanism of action become more fully understood, its application will become more refined, with more appropriate usage of this valuable tissue. However the full potential of the tissue is not known and further research is required. With any surgical procedure, AMT has presented complications and side effects

Table 1. In some cases it is not possible to separate complications from the tissue itself or from complications arising from surgical procedures or techniques [12, 61].

List of complications	***Complications from tissue itself/donors***	***Complications from surgical technique***	***Complications from recipient***
Post-operative infection	+	+	+
Dislocate as a result lose/broken sutures	+	+	+
Haemorrhage under the membrane	-	+	+
Early disintegration of the AM	+	+	+
Limitation (availability)	+	-	-

Table 1 List of complications with AMT. Table show the list of common complications with AMT. Complications divided into three main groups according to the origin such as donor/tissue complications; complications during surgical technique: recipient/patient complications. [172]

1.6 Biochemical composition of AM

AM has been widely used for ocular surface reconstruction and its biochemical composition is well established [173].

A plethora of growth factors and cytokines have been identified in AM and amniotic fluid [174-178]. These are a positive attribute of the membrane, which make it effective in surgical applications. Important factors, which promote wound healing, include transforming growth factor (TGF)-β [179, 180] epidermal growth factor (EGF) and matrix metalloproteases (MMPs) [181-183]. However, it is not yet clear whether these molecules survive the processing and storage techniques employed to preserve the tissue.

1.6.1 Growth factors

Growth factors are responsible for cell proliferation and differentiation and can simultaneously play a positive or negative role in wound healing.

AECs have been shown to be a potential origin of growth Factors such as EGF, TGF-α, keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), basic fibrinogen growth factor (bFGF), transforming growth factor (TGF)-β1 and TGF-β2. Many of these factors have been detected in AECs at the mRNA and protein level [184].

TGF-βs are known to stimulate the synthesis and deposition of extracellular proteins and they are involved in the mediation of fibroblast activity during wound healing and the scarring process. Presenting TGF-β to defects in the ocular surface during AMT, increases fibroblast activity and increased scarring

1.6.2 Cytokines

IL-6 and IL-8 are found in high concentrations in AM and amniotic fluid (AF) [61]. These cytokines are associated with labour, and they are also produced in response to infection [185-187]. IL-6 and IL-8 are thought to be involved in the inflammation of AM, especially during parturition, and are detected with increasing levels towards term, in both labouring and non-labouring patients. In addition both have been shown to inhibit AEC proliferation [186].

Moreover, the pro-inflammatory cytokine IL-18 was detected in AF [188, 189], therefore it is constitutively expressed by the AM and may be involved in response to microbial invasion of the amniotic cavity.

IL-1α, IL-1β and IL-1 receptor antagonist have been reported in AM and are thought to be endogenous mediators of inflammation and parturition. These cytokines are associated with the production of IL-6 and IL-8 during intrauterine infection and activation of the labour process in a cascade of inflammatory events [187, 190]. Human interferon gamma (IFN-γ) is unique to AM and is classified as

a novel sub-type 1 interferon [191]. AM IFN-γ is antigenetically unrelated to IFN-α, -β or –γ, however it shares similar biological activities to IFN-α and IFN-β.

1.6.3 Angiogenic factors

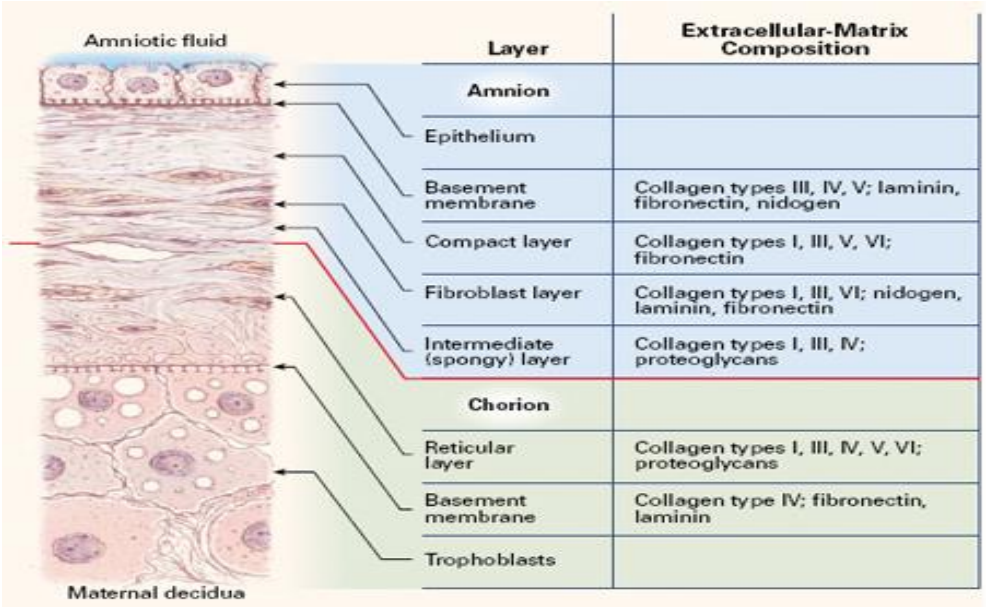
Angiogenesis is the formation or “budding” of new capillaries from pre-existing vasculature, by migrating and proliferating endothelial cells [192, 193], Combined with the process of vasculogenesis, it is known as neovascularisation. Physiological angiogenesis occurs during embryonic development, the menstrual cycle, muscle development, increasing activity which appear to have more mechanical signals including increased friction on the inside of blood vessels and stretch of vessels caused by the surrounding muscle fibres. In pathological conditions such as wound healing, tumour growth, psoriasis and diabetic retinopathy cells signalling to increase function and forms new blood vessels, AMT in any situation and using any methods (graft or patch) promoted reepithelialisation, decreases inflammation and fibrosis and inhibits angiogenesis [61, 194].

1.7 Spongy Layer

Little is known about the structural composition or function of the SL. The SL is thought to possess similar properties to Wharton jelly (WJ) [48, 195-197]. Both tissues types have very high hydrophilic properties with a hydrated water absorbance of 98% and they are highly durable minimising mechanical damage. It has been shown that WJ has a limited number of cells, but this has not been confirmed in SL. The percentage of extracellular matrix (ECM) components in WJ is high with an abundance of collagen, glycosaminoglycans, hyaluronic acid and several sulphated proteoglycans. Though lower in density, the expanse of collagen makes SL resistant to extension and compression evoked by fetal movements and uterine contractions [195-198]. The ECM in SL also contains a large amount of fibroblast growth factor (FGF) [47, 48, 199].

1.7.1 Structure of Spongy Layer

Spongy Layer, is a wavy layer [200], compressed between amnion and chorion during process of development, embryology. This layer contains a few isolated fibroblasts [201], and Hofbauer cells [202]. (Figure 1) and (Figure 7). These cells are possibly remnants of the original amnionic or chorionic mesothelium [203]. The thickness of the SL is a highly variable [203], SL may vary between 5µm to 10mm [202] Collagen IV (Figure 7) presented in the SL more likely to be mesothelial origin as it is a typical basal lamina molecule, from theory Malak et al [45] and Ockleford et al [41, 200]. Schmidt et al reported macrophages in the SL [176].



Layer	Extracellular-Matrix Composition
Amnion	
Epithelium	
Basement membrane	Collagen types III, IV, V; laminin, fibronectin, nidogen
Compact layer	Collagen types I, III, V, VI; fibronectin
Fibroblast layer	Collagen types I, III, VI; nidogen, laminin, fibronectin
Intermediate (spongy) layer	Collagen types I, III, IV; proteoglycans
Chorion	
Reticular layer	Collagen types I, III, IV, V, VI; proteoglycans
Basement membrane	Collagen type IV; fibronectin, laminin
Trophoblasts	

Figure 7 Cross section of the human amniotic membrane with biochemical characteristics of the section. Image belongs to: https://www.netcells.co.za/membrane-about.php the figure shows the layers of the amnion and the chorion with some biochemical characteristics of the layers.

1.7.2 Composition of the Spongy Layer

Little is known about then biochemical composition of the SL. Recently, TGF-b1, EGF and HGF reported to be present in SL by Hopkinson et al in 2006 [111] and Koizumi et al [184].

Considering the embryological formation of the spongy layer and its function as physical boundary between the cellular layers of the vascular chorion and avascular amnion, the potential biochemical composition of the SL is vast.

Amnion consists of fibrous connective tissue with a high concentration of collagen. High levels of hyaluronan are present was in the decidual cell layer. [27, 28]. Meinert *et al* [204] reported, that SL has intense stained with hyaluronan, compare to chorion with small amount of hyaluronan stain and amnion with high concentration of hyaluronan, Amnion was dominated with decorin and chorion with biglycan.

However, the effects of SL on cell growth and differentiation needs to be established.

1.7.3 Physiology and function of Spongy Layer

The surface of opposing amnion and chorion fibroblastic layers appear as complex ridge-like folds [39], which are propose to interlock in gyri and sulci formation to provide stable connection between the connective tissue layers. The SL forms between these layers and although the precise *in vivo* function is unclean, is hypothesised to have several functions:

i) provides mechanical support for the amnion and chorion;

ii) exchange of the nutrition and waste material between fetus and mother;

iii) production and secretion of hormones and immunological barrier.

Providing mechanical support, the SL provides a platform that allows the amnion to slide upon the chorion, which is firmly adherent to the maternal decidua [201]. Several reports have linked dramatic changes in the mechanical properties of the fetal membrane and the SL prior to delivery, as swelling and changing hormonal phone to induce delivery where oxytocin plays key role and together with prolactin and oestrogen release prostaglandins, which play a role in softening and ripening cervix [204, 205]. They think, that membrane rupture could be due to interaction between collagen and decorin. As hyaluronan have the ability to swell significantly, it can lead to separation of the two layers and decrease mechanical strength, as those layers will not be able to work in parallel any longer [204].

The chorion and the amniotic fluid transfer nutrients to the avascular amnion by diffusion [206]. If the chorion and amniotic fluid transfer factors by diffusion all those factors passing through the SL and may have influence on SL protein profile, if SL will accumulate those factors. As amniotic fluid (AF) originally forms from maternal plasma through fetal membranes by osmotic and hydrostatic forces [207, 208].

One is that the SL provides a buffer zone to allow the amniotic layer to slide over the chorion. It is also thought that the SL causes dramatic changes in the mechanical properties of the foetal membrane [196, 209]. The mechanical

properties of the foetal membrane have been extensively studied and it has been shown that, the biochemical properties of the foetal membrane are change prior to delivery [30] and foetal membrane in cervical area became thinner, possible due to mechanical micro rupture, which allows AF goes through AM, imbibes by SL, last swells and giving extra power for rupture. Additionally, JJ Giquel et al has shown that amnion thickness varies between placental, apical, central and cervical regions.

1.7.4 Our observations on the SL and why it became a focus of study

SL is the layer at which the amnion and chorion separate when preparing AM for clinical application. Work by Dr Hopkinson, in my department discovered several key inconsistencies in preparing AM for transplantation, during which the SL is often overlooked and ignored. Typically, the fetal membrane is collected whole (Figure 9. A). The reflectum (sac) region is isolated from the placenta (Figure 9 C). The AM is then isolated from the fetal membrane by blunt dissection from the chorion of the reflectum (Figure 9 E) and the placenta (Figure 9 D). This results in two separate pieces of AM, with an exposed SL (Figure 9 F). However, our observation is that the SL may remain associated with the chorion or AM, or partially on both. Therefore, current transplant ready AM (TRAM) may or may not have SL. This immediately produces variability by not standardising the processing of the membrane and may have unknown clinical effects. Figure 8.

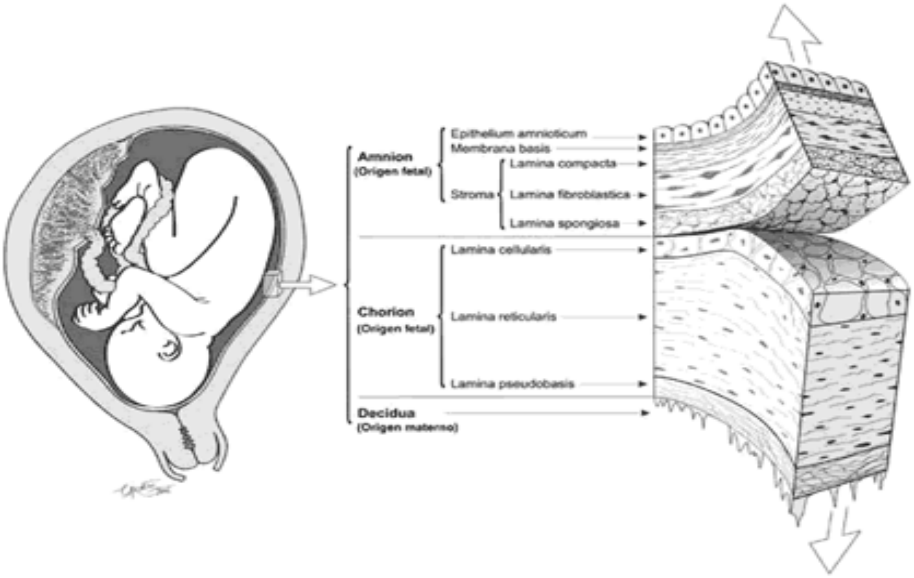


Figure 8 Separating amnion from chorion. Image belong to: https://www.netcells.co.za/membrane-about.php. Showing area of the separation amnion from the chorion in spongy layer zone.

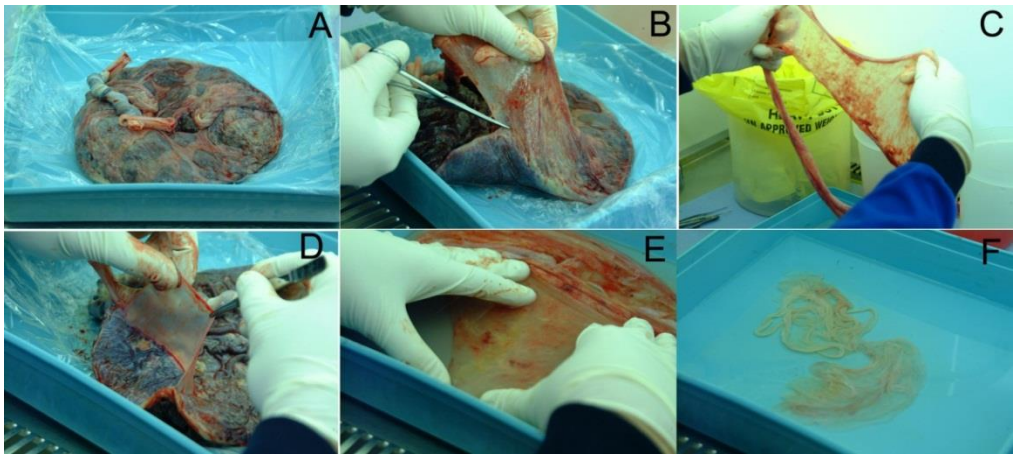


Figure 9 AM isolating technique.
Showing AM isolation technique. (A) the placenta received from obstetric department; (B) cutting foetal sac (amnion and chorion attached); (C) all foetal tissue separated and ready to be washed and processed; (D) separating the placental amnion puling with scissors from placenta; (E) foetal sac washed and ready to separate amnion from chorion; (F) amnion detached from the chorion and waiting to be wash.

Once isolated from the fetal membrane the conventional process for removing blood from the AM is to repeatedly wash the AM with gently rubbing to remove any remnants. However, this has two negative effects. The first is that because of the 'sticky' nature of the SL, the majority of visible staining is blood sticking to the SL surface (Figure 9 C). Blood sticking to the epithelial side of the AM tends to wash away freely. With initial rubbing of the SL, blood is integrated into the SL (Figure 9 F) making complete blood removal difficult. This requires more

extensive washing and 'rubbing' to remove residual blood, resulting in desegregation of the SL (Figure 9 C). The end product is a membrane with patchy and inconsistent SL retention with occasional persistent discoloration from blood deposits (Figure 9 C). The diagram Figure 10 shows the same process described above and shows remained blood attached to SL.

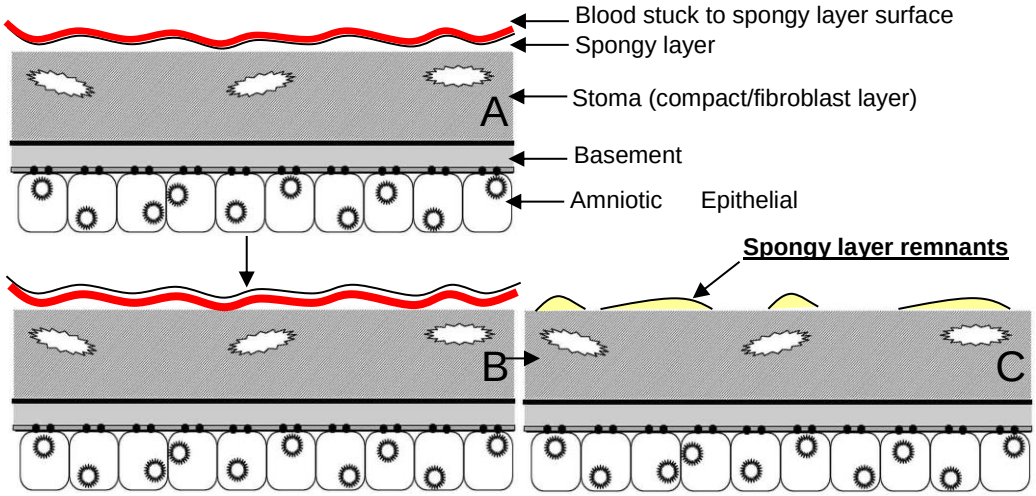


Figure 10Traditional AM isolation technique.
A diagram showing traditional processing of AM to remove blood contamination. (A) represents initial AM with blood attached to the SL surface; (B) represents AM after mild washing and rubbing pushing blood deposits further into the SL; (C) represents AM after processing prior to preservation, showing disaggregated and heavily patchy SL impregnated with residual blood contamination.

Processed membranes then undergo a quarantine process for a minimum of 6 months prior to transplantation and experimental analysis. Before use, the surgeon or scientist typically washes the membrane, often removing any visible excess SL debris. However, in our experience, once the SL has been mechanically disaggregated and subjected to a preservation step, the structural integrity of the SL is partially lost, such that it becomes almost impossible to fully remove. Therefore, it is inevitable that membranes used clinically or experimentally retain regions of residual SL.

The preliminary work by Dr Hopkinson suggested the consequence of this might be transplanting contrasting and potent biochemical and ultimately functional properties to the ocular surface. To overcome these potential issues, Dr Hopkinson developed a standardised AM preparation technique to reliably

remove the SL intact during processing. This procedure is now accepted for clinically preparing membranes for human application, SL free. The composition and function of the SL is yet to be further explored.

The concept for this study was therefore to develop further technique of preparing the tissue for clinical usage.

With this in mind, the hypothesis was to look at SL as a new treatment solution in therapy of ocular surface disorder, where SL can be beneficial in term of antimicrobial activity and wound healing.

The test this hypothesis, the overarching objective was to understand embryological origin of the SL and biochemical composition and structure of the tissue and it effect on corneal epithelial cells. To achieve this objective the research was separated into clearly defined aims set out as chapters.

To date, there is no previous ophthalmic research literature describing the use of AM specifically with or without spongy layer. Tseng reference the SL on two occasions. The first was in his patent “Grafts made from amniotic membrane; methods of separating, preserving, and using such grafts in surgeries”, acknowledging the SL as part the anatomical structure of the AM. However, although his patent describes the preparation of the AM, there is no mention of removing or preserving the SL. The second was in a manuscript, where it says the AM and chorion are fused and that the ‘spongy layer’ is the space generated during processing [80]. This is not accurate as the SL occupies a space between the layers [210], but may expand further during processing

Acknowledging the SL as a vast potential source of a broad spectrum of unknown factors, and the potential subsequent clinical variation, presented by the non-standardised membranes prepared using conventional techniques, we exploited the hydroscopic properties of SL to develop a highly effective method to standardize AM processing.

AM is isolated from the fetal membrane and, rather than the standard washing and rubbing steps, membranes were transferred immediately to saline and with

agitation on a rocker and without any mechanical rubbing to remove the blood. The wash buffer was changed periodically until all visible blood contamination had been removed.

Without mechanical intervention (rubbing), the blood contamination gradually reduces until no blood is visible. At the same time the SL swells, typically 3-5 times its normal thickness, until the layer can clearly be seen (Figure 11 & Figure 12 A-C). The AM is then outspread, SL side up, and the reverse edge of a scalpel blade is used to break through the SL making sure it does not penetrate into the stroma of the AM (Figure 11 A & Figure 12 A). A tear is generated in the SL right across the AM surface and then the scalpel blade is used to gently pull the SL back away from the AM layer (Figure 11 B & Figure 12 B). It is important here distinguish between 'pulling' and scraping of the SL. Scraping the SL disaggregates the layer removing only the surface of it and therefore not removing the SL completely. When the SL is thick, forceps can be used to gradually pull the SL completely away from the AM layer (Figure 11C-D and Figure 12 C-D)

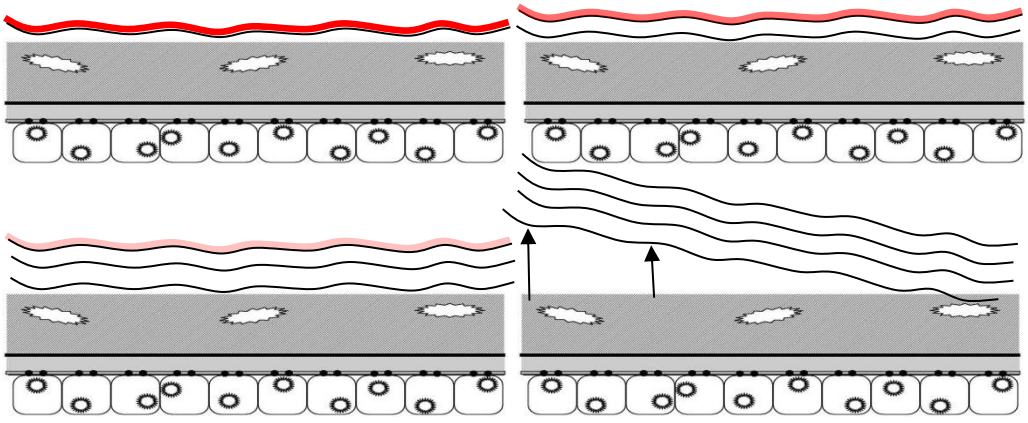


Figure 11 Novel method of complete removal of the SL.
Diagram illustrating our novel method for complete removal of SL. (A) represents standard blood contaminated isolated AM; (B) washing without mechanical intervention begins to elute surface blood contamination and the SL begins to swell; (C) continued washing removes blood further and the SL continues to swell; (D) eventual blood removal is followed by removal of the expanded SL.

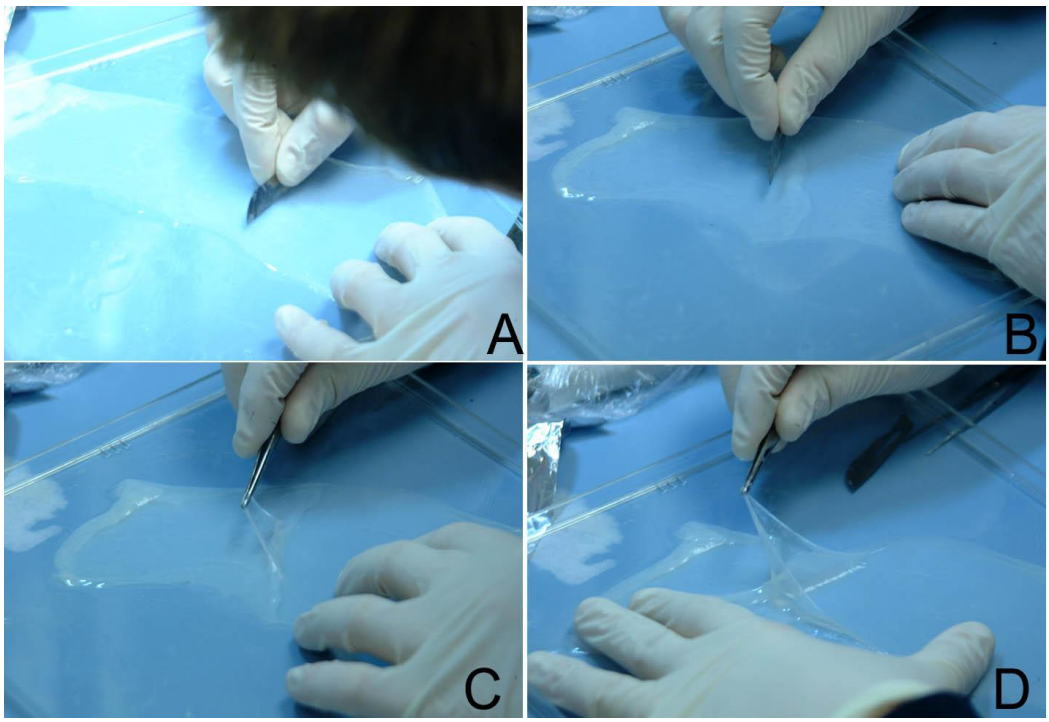


Figure 12 Stages of SL removal.
Images depicting different stages of spongy layer removal. (A) the amnion after wash SL side up, gentle cutting of the SL with not sharp side of the blade without cutting amnion; (B) pushing the SL to separate from the amnion; (C) pulling the SL with forceps; (D) by pulling SL and holding the amnion at the same time is clear to see a layer shape of the SL.

To validate the effectiveness of SL removal, we have assessed membranes prepared using our novel procedure and conventional procedures, using scanning electron microscopy (SEM). Under SEM, an intact SL appears as a fine network of loose fibres that appear light in colour (Figure 13 A). After partial removal of the SL by gentle scraping, areas of SL-related fibres remain (Figure 13 B, white fibres) with an underlying densely organized collagen type I ECM (Figure 13 B).

Some damage to the collagen type I organization can be seen following scraping. When prepared using our novel procedure, SL related fibres have been completely removed, exposing an undamaged and dense collagen type I ECM (Figure 13 C). After preparing membranes using conventional procedures, patches of SL can still be seen and is highly disaggregated (Figure 13 D).

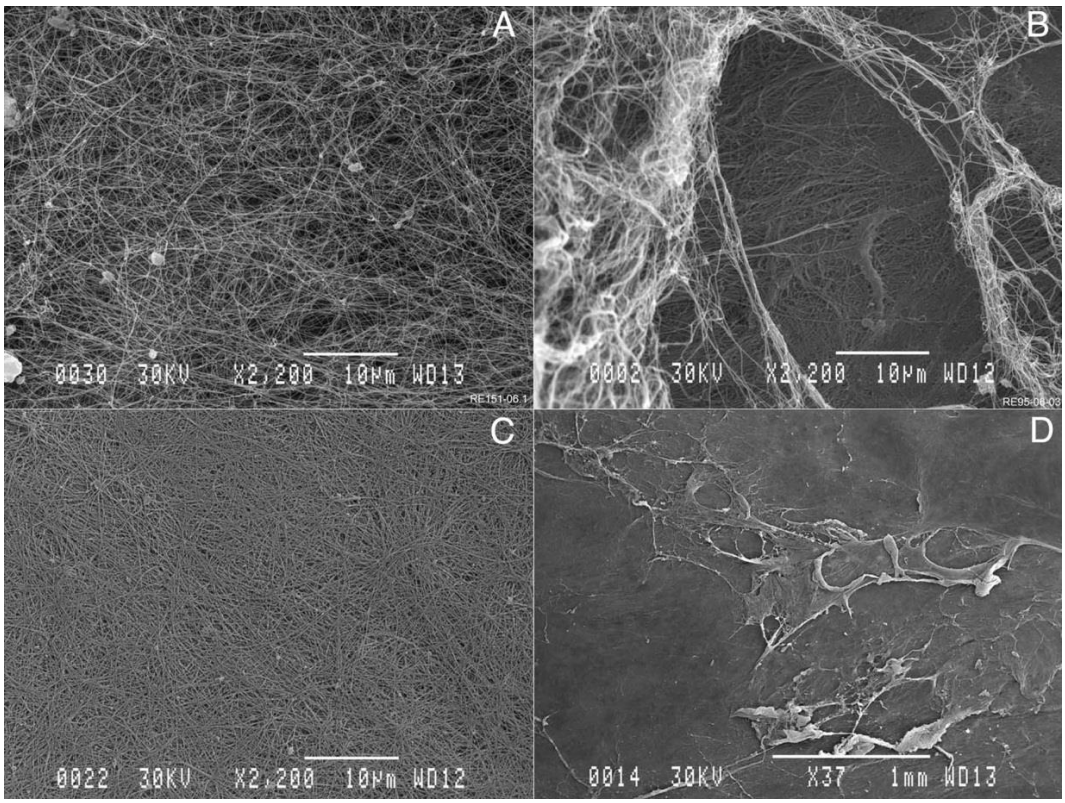


Figure 13 SEM of the SL.
SEM of the SL, (A) intact on the AM;(B) after partial removal by rubbing and scraping; (C) completely removed using our novel technique; and (D) after preparation using conventional techniques.

1.8 Aims

1. Identify the embryological origin of the Spongy Layer from the literature review and from available sources for the research.
2. Investigate biochemical compositions of the Spongy Layer as an independent layer.
3. Establish and optimised separation of the proteins in the SL and fractionation of those proteins to the standard level.
4. Identify key proteins contributing to the mediation of wounding and scaring by SL treatment.
5. Investigate effect of SL on human CEC, KFB, and lymphocytes.
6. Identify key factors responsible for cells proliferation and cytotoxicity.
7. Investigate antimicrobial effect of SL.
8. Investigate differences and similarities between TRAM and SL.

1.9 Objectives

In my project, I would like to prove that the SL is a separate layer, which has undeservedly been ignored during AM preparation. This layer has significant amount of growth factors compared to AM itself. The SL has been shown to accommodate a complex proteome, which enclose many water-soluble factors.

Main purpose of the study was to establish the biochemical composition of SL, as the tissue was completely ignored and biochemical composition of the SL has not been established.

Chapter 2 – The Independent Embryological development of Spongy Layer

2.1 Introduction

Bourne and Wiese were the first sciences, who described Spongy Layer in 1966 [209] and 1975 [211]. However, this layer has been reported by different nomenclature including a hyaluronan-rich gelatinous substance [28], intermediate layer [212, 213], human amniotic jelly [118], jelly-like layer [214], spongy coils [45], extraembryonic coelom [215] and inner avascular amnion. [216-218]. From literature, this layer less structurally defined gelatinous, protein-rich and known as the extraembryonic coelom or spongy layer (SL) [31, 201, 209, 219, 220].

Mechanical separation of the amnion and chorion typically results in the SL remaining associated with the AM. However, the AM preparation technique developed by Hopkinson [42, 111, 221], clearly indicates the SL can be easily dissociated from the AM following swelling *ex vivo*, and can be completely isolated from the membrane. The SL can be mechanically peeled from the AM, attached only by fine fibrous connections (Figure 9 and

Figure 10). Because of the clear differences in structural properties of the SL to the dense ECM of the AM and chorion, and the ability to separate the SL from both membranes, the theory was formed that the SL may actually be a developmentally distinct layer to the AM.

2.1.1 Embryological development of the SL

As conventionally, the SL is considered to be the inner most layer of the AM, [31, 201, 209, 222], it is therefore assumed that the SL develops as part of the AM during gestation. This evidence suggests therefore that the SL may, in fact, not be a compositional layer of the AM, though the tissue may produce this during gestation.

To investigate this further a comprehensive literature review was performed to in an attempt to elucidate the true embryogenesis of the SL. It was concluded that The SL develops as a separate independent layer between day 6 and week 12,

(Figure 14) during the period of gestation in the chorionic cavity, when the AM fuses with the chorion, compressing the loose ECM into a compact gelatinous SL[223].

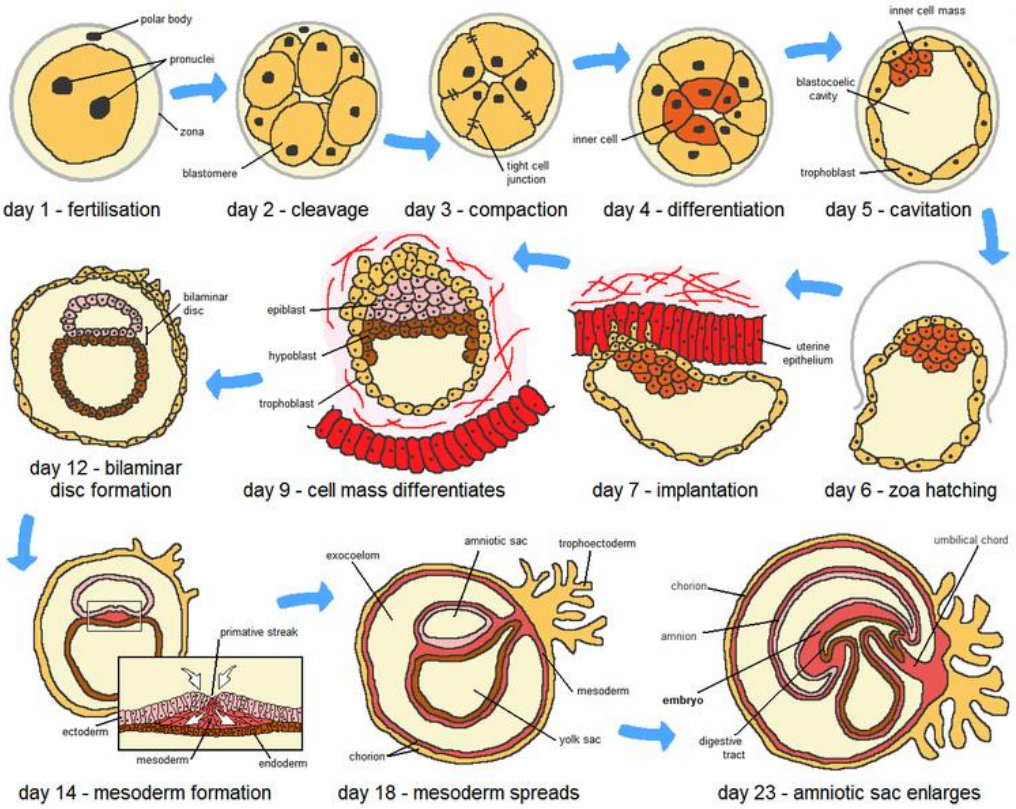


Figure 14 Development of fetus and AM day 1 to day 23 of gestation. Development of the foetus and AM layers from day 1 to day 22 of gestation.http://commons.wikimedia.org/wiki/File:HumanEmbryogenesis_cs.svg Diagram shows, how the amniotic sac forms and expands, main while compressing exocoelom, which forms a spongy layer.

Explaining SL development in more detail, by following the Figure 14

i) Between days 6-7 the blastocyst attaches to the uterine wall and implantation of the embryo commences;

ii) Cytotrophoblast cells proliferate and begin to invade the uterine epithelium, which is achieved through digestion of the uterine cells by secretion of enzymes by the cytotrophoblast cells. Upon contact with the endothelium the contacting cytotrophoblasts form the syncytiotrophoblasts;

iii) At day 12, Figure 14 the syncytiotrofoblasts surround the cytotrophoblast cells of the blastocysts. On the border between the blastocoele and the

inner cell mass (ICM), 2 layers form the epiblast and hypoblast. The epiblast layer is composed of tall cells forming the floor of the amniotic cavity (primitive ectoderm). Epiblasts migrate around the outer edges of the extraembryonic reticulum and form the extraembryonic mesoderm, which makes it difficult to support the extraembryonic reticulum;

iv) Subsequently, this causes spaces or pockets to form in the extraembryonic mesenchyme (days 12-13) (Figure 14), which expand and coalesce to form a single cavity of extrambryonic coelom (chorionic cavity). This cavity then becomes occupied with chorionic fluid. The amnion remains attached to the chorion at a connecting stalk, and the extraembryonic coelom separates somatic (lines trophoblasts and amnion) from splanchinic (around primary yolk sac) extraembryonic mesenchyme. The primary yolk sac decreases in size, Figure 14shows primary yolk sac on day 12 of gestation taking majority of the volume of the embrion, which significantly reduced in size by weeks if gestation period –Figure 15A and very small size of primary yolk sac closer to birth Figure 15 B;

v) On day 18, Figure 14 can see clearly, these cells grow around the ICM to form the amniotic cavity, lined by amnioblasts. The hypoblast is closest to the blastocyst cavity, and is comprised of short cells. These cells migrate along the inner lining of the inner cytotrophoblast lining of the blastocoele, secreting extensive ECM along the way. These cells and the ECM are called the Heuser's membrane or exocoelomic membrane, and the blastocoel is now the primary yolk sac (primitive endoderm) or exocoelomic cavity [220, 223] Between days 14 and 21 the expansion of the amnion compresses the chorionic cavity and the extraembrionic mesoderm, until the amnion and chorion fuse (Figure 15).

vi) Cytotrophoblast cells and cells of the Heuser's membrane (hypoblasts) continue secreting ECM. This matrix is called extraembryonic reticulum

(now considered pre-SL). This pre-SL pushes against the primary yolk sac causing it to shrink within the exocoelomic cavity [210, 224], day 23, Figure 14.

vii) Compression of the extraembrionic somatic mesoderm and extraembrionic reticulum form the SL. As previously mentioned from a developmental process, SL is a separate independent layer.

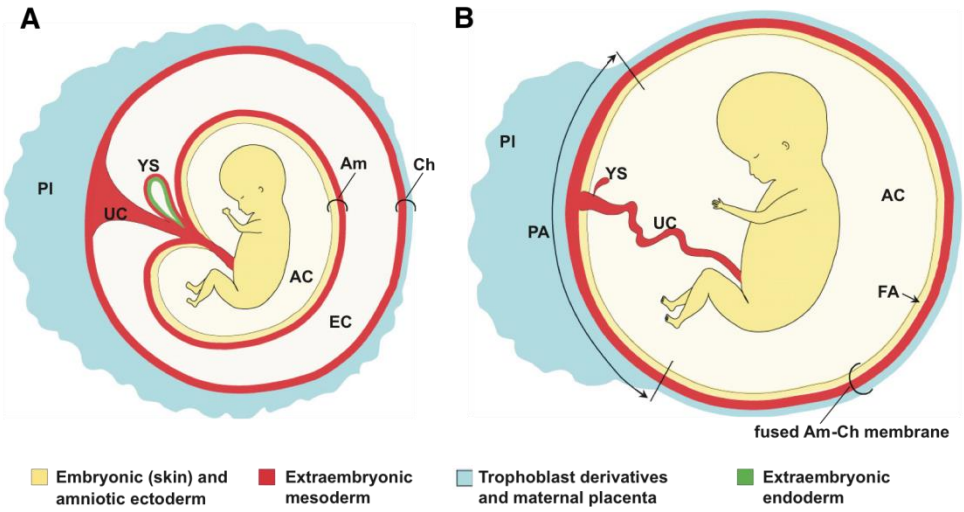


Figure 15 Thickness of an exocoelom layer (EC) and yolk sac in the early stage of the development.
An image illustrating a thick exocoelom (EC) layer and yolk sac in the early stages of development. With increasing gestation age the EC layer becomes compressed and forms the thin layer termed the SL. Dobreva et al Int. J. Dev. Biol. 54: 761-777 (2010).

AM forms the amniotic cavity, the first among the three embryonic cavities (amnion, chorion and yolk sac) to develop. The amniotic ectoderm or the roof of the amniotic cavity is comprised of a single stratum of flattened cells, and forms from the prismatic ectoderm, of the embryonic disc. The amniotic ectoderm is covered by a thin layer of mesoderm, which is continuous with that of the somatopleure and is connected, via the body-stalk, to the mesodermal lining of the chorion. The somatopleure is a combination of ectoderm and mesoderm and gives rise to both the amniotic and chorionic membrane. Ectodermal tissue supplies functioning epithelial cells, and the mesoderm generates the essential blood supply to and from the epithelium. The AM forms as a layer of epiblast cells

that expand towards the embryonic pole and differentiates into a thin membrane that separates the new cavity from the cytotrophoblast and eventually constitutes the lining of the amnion (appears on day 8 of foetal development).

The amniotic fold is formed when the somatopleure folds upwards and at the point of constriction where the primitive digestive tube of the embryo joins the yolk sac. The fold tip meets and fuses over the dorsal aspect of the embryo, forming the amniotic cavity. The two layers of the fold then become completely separated, the inner forming the amnion, the outer forms the chorion. The space between the amnion and the chorion constitutes the extra-embryonic coelom. When the AM has formed, amniotic fluid begins to accumulate within the cavity between the fourth and fifth week of gestation. This increases in quantity and causes the AM to expand and to ultimately adhere to the inner surface of the chorion.

2.2 Methods

2.2.1 Ultrasound measurement of SL. SL thickness

It is difficult believe, however that scientists started to measure the distance under the water using sound waves in 1826 in Switzerland, in medicine the ultrasound started to be used in 1920 in Germany, but not as diagnostic technic, as a therapeutic to hit tissues [225], [40]. As a diagnostic tool the ultrasound first was used in 1950s in USA and only in 1960s in obstetrics and gynaecology in USSR. Nowadays, this technic is very well known and established. Novelty of our usage of the ultrasound technic is only to measure the SL as a separate layer out of amnion and chorion. This idea came after measurements of amniotic membrane in vitro and established difference in thickness [113].

Methods:

The investigate thickness of the SL their role was a part of the PhD thesis work at the University of Nottingham UK. The measurement of the thickness of the SL in vivo was one part of the project. To avoid technical mistakes in measurements, very experienced sonographer particularly in foetal-maternal medicine reformed all scans.

Research participation:

Verbal consent was obtained from 25 women in the department of Foetal-Maternal medicine (FMM) in the University Hospital of Nottingham NHS Trust, who came for a routine ultrasound scan. During the examination only two extra measurements were carried out. The thickness in the placental and cervix areas [226]

Data expression

All results were divided into groups according to the gestational age, patient's age. All patients, selected for the participation were healthy with no diabetes diagnosed and normal BMI [Girolami, 2005 #116].

Ultrasound machine Voluson 730 from GE Healthcare been used for ultrasound examination, which give good resolution and image in 2D and 3D vision. All results were divided into three groups according to gestational stage and SL thickness calculated using Microsoft Excel 2010.

Statistical analysis

All statistics were analysed with use of SPSS for Windows and Microsoft Excel 2010. For comparison between three groups, were performed using the nonparametric Mann-Whitney U test and $p<0.05$ was considered significant.

Measurements of SL thickness was performed by an experienced sonographer in the department of FMM University Hospital of Nottingham NHS Trust. Verbal consent was obtained from all 25women with different (20 – 37 weeks) of gestation age, which participated in the project, assessed for SL thickness. During routine ultrasound scan appointment, one extra measurement set was carried out. The SL thickness measured in placental area (area of amniotic membrane attached to placenta body) and cervix area (area of potential rupture zone) [227, 228]. Ultrasound machine Voluson 730 from GE Healthcare been used for ultrasound examination, which give good resolution and image in 2D and 3D vision. All results were divided into three groups according to gestational stage and SL thickness calculated using Microsoft Excel 2010.

2.2.2 Comparison WJ and SL

From close look at the placenta, it seems, that placental amnion, the part of the amnion, which cover the placenta, continues to the umbilical cord, as seen on the

photo Figure18 A. The trypan blue been injecting into WJ and was passing through to SL, however, only in few samples it was possible. WJ and SL are the different tissues with similar structure and properties.

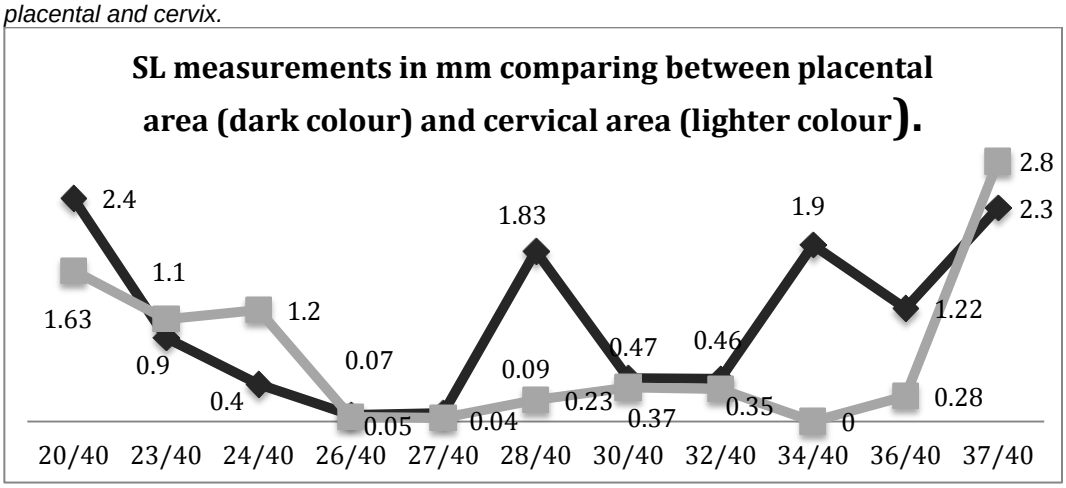
2.3 Results

Results presented show difference in thickness in two different areas (placental and cervical) measured in 25 patients. The age range was between 21 to 42 years. The gestational rage was between 20 to 37 weeks. And this was divided into 3 subgroups, early gestational age (20-23 weeks), mid gestation (26-30 weeks) and late gestation (30-37 weeks) Subgroups 20 -23 weeks had a smaller fetus (weight between 300 – 500 g), which allow to see fetal membrane with a distance from the fetus, second subgroup (weight of fetus between 700 to 1500 g), and third subgroup (weight between 1500 to 3000g), baby taken nearly whole space in uterus. First and second subgroups are the second trimester of the gestation, divided according to the fetus size. The third subgroup, is the third trimester of the gestation. Taking measurements from the placental region was straightforward, but obtaining measurement near the cervix area was difficult due to anatomical variations and location of the cervix against the sac. Due to that fact 8 women were excluded from measurement of the SL thickness in the cervix area, 1 out of 8 excluded women had twins

Table 2.

Patient number	*Period of gestation*	*Thickness of SL (placental part)*	*Thickness of SL (cervix area)*
1	20/40	1.1mm	1mm
2	20/40	1mm	Twins
3	20/40	0.7mm	5mm
4	20/40	0.5mm	0.5mm
5	23/40	0.9mm	1.1mm
6	24/40	0.4mm	1.2mm
7	26/40	0.05mm	0.05mm
8	26/40	0.09mm	0,05mm
9	27/40	0.09mm	0,04mm
10	28/40	0.7mm	0.7mm
11	28.4/40	1.2mm	n/a
12	28.5/40	1.4mm	n/a
13	30/40	0.7mm	0.5mm
14	30/40	0.6mm	0.3mm
15	30.5/40	0.1mm	0.3mm
16	32/40	1.8mm	0.5mm
17	32/40	1.2mm	0.2mm
18	34/40	1.9mm	n/a
19	36/40	2.4mm	1.2mm
20	36/40	1.6mm	n/a
21	36/40	1.8mm	n/a
22	36.3/40	1.8mm	n/a
23	36.5/40	1.3mm,	n/a
24	36/40	1.8mm	0.45mm
25	37/40	2.3mm	2.8mm

Table 2 Ultrasound measurement of the Spongy Layer. Table shows the number of women participated in the research project and measured thickness of the SL in two different areas: placental and cervix.



Looking on

Table 3 and Figure 16, thickness of SL was more than twice as thick (Significance value) in the cervix region than the placental region in the earlier stage of gestation. However as gestation progresses, the SL thickness in cervix

region decreased, whilst the thickness in the placental region significantly increased to 1.7 times that of the cervix region. Ultrasound measurement can be seen on the images Figure 17, where SL clear to see in placental areas and need to be experienced sonographer to be able to measure SL in cervix area.

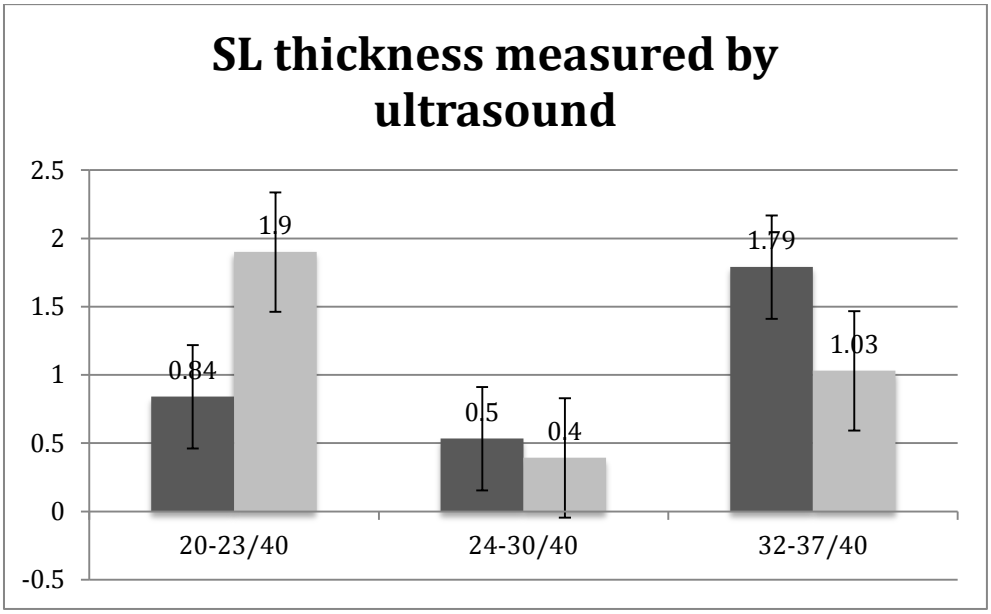


Figure 16 SL thickness measured by ultrasound This figure shows the thickness of the SL in placental area (dark colour) compare to cervix area (light colour), results mirrored from the Table 4. All measurements perform in mm.

Age of gestation (weeks)	***SL Thickness (mm)***	
	Placental	***Cervix***
20-23	*0.84 (n=5)*	*1.9(n=4)*
26-30	*0.5(n=10)*	*0.4(n=8)*
32-37	*1.79(n=10)*	*1.03(n=5)*

Table 3 Ultrasound measurements of SL thickness.
Thickness of SL in three different ages of gestation period in two measurement areas. Shows in cervix area in second and third trimester of pregnancy, SL thinner compare the SL thickness in placental area. And in placental area SL thicker close to delivery date.

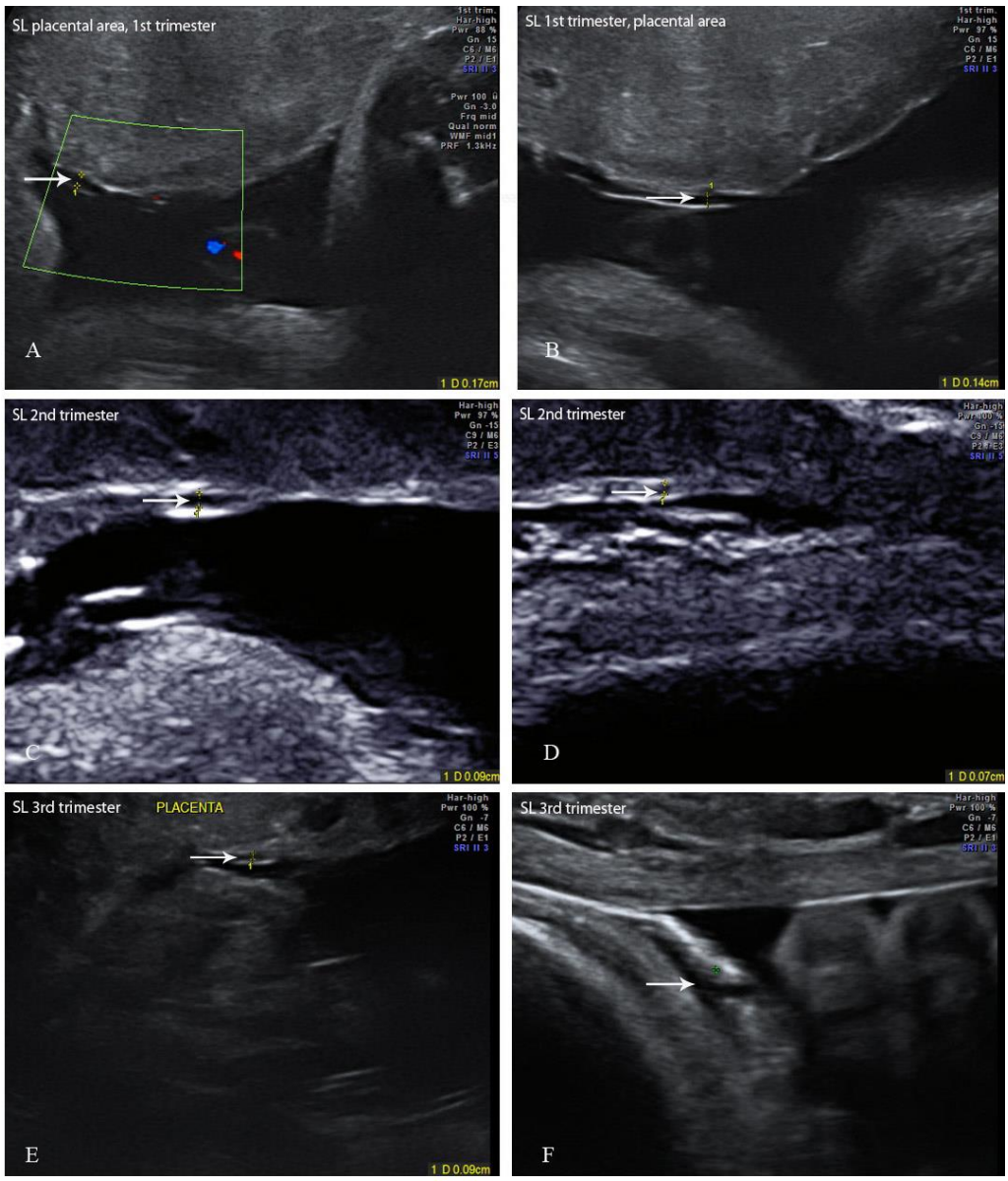


Figure 17 SL ultrasound measurement.
SL thickness measured by ultrasound (A) first trimester of pregnancy, cervix area, thickness of SL is 0.17 cm (arrow); (B) first trimester of pregnancy, placental area, SL thickness is 0.14

It was thought, that was the possibility of SL and WJ to be identical tissue, as from the literature, WJ is a gelatinous tissue with hyaluronic substrate [229, 230]. The SL extends to the umbilical cord along with the AM layer to become Wharton's jelly, which is against the embryological founding of the SL and WJ. From embryology Figure 17 SL is a layer of extracoelom, developed from blastocoelic cavity, which compressed between chorion and amnion during the development. The WJ is a primitive streak, developed from trophoblasts, in the

place where blastocyst become attached to uterine epithelium. I used trypan blue to inject into WJ to see connection between SL and WJ.

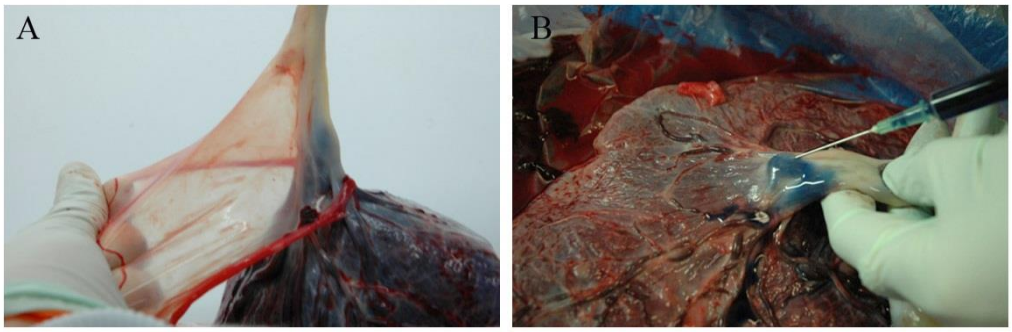


Figure 18 Connection between the AM and WJ. Figure (A) shows the connection between WJ and AM, placental part of the AM continues to the WJ; (B) shows injection of Trypan blue into WJ and it goes to SL, the layer between AM and chorion.

2.4 Discussion

The results indicated that the spongy layer is at its thickest in the cervix region at the early stages of gestation which then becomes thinner as gestation progresses.

Which can be against theory of Malak and Bell [48] that the cervical membrane increased in thickness in the connective tissue and decreased in cytotrophoblasts layer. ECM proteins synthesized by several cell types within the amnion and chorion giving the strength and elasticity of the fetal membrane, prior to the labour, those proteins regulated by MMPs and their inhibitors, which reduce elasticity [231].

Our findings support our hypothesis that the SL develops independently to the AM. SL material is formed in the fetal membrane sac region between the amnion and chorion in the first trimester of pregnancy routinely described extraembryonic coeloms. Then, as the foetus develops and the amniotic sac expands, it progressively becomes compressed the chorion, which also compresses the extraembryonic coelom, decreasing the thickness of the SL until the point at which the material is maximally compressed in the third trimester [232] [233].

On the other hand, the placenta area is where all layers are attached at the base of the cord. This means the extraembryonic space starts minimal in the first trimester as seen in our data.

If this theory is correct, as I believe so, and theory about micro rupture in whole amniotic membrane prior to end of the gestation age, SL swell by inhibiting AF from the fetal sac, which can be interesting potential investigation.

Some researchers suggest that disruption between amnion and chorion prior to delivery is due to release of phospholipases, eicosanoids (prostaglandin E_2), cytokines, elastases, MMPs [234].

Chapter 3 – Biochemical Composition of The Spongy Layer as an Independent Layer.

3.1 Introduction

AM is well-known surgical adjunct successfully used in ocular surface reconstruction (described in Chapter1). The combination of anti-inflammatory, anti-microbial and antiviral, anti-fibrosis, anti-scarring and anti-angiogenic properties [18, 34, 36, 83, 177, 235, 236] of the AM makes this tissue unique for the surgeons and researchers. The extracellular matrix components of the basement membrane of the AM create an almost native scaffold for the cell seeding in tissue engineering [177]. The Amniotic Membrane Transplantation (AMT) has different outcomes and significant clinical variations.

Due to the lack of understanding regarding the SL, clinically this layer has either been ignored during processing resulting in the variable disruption and removal.

From all that points, if biochemical properties of the SL is the same or very similar to the biochemical properties of the AM, this investigation may have two different outcomes: a) SL may be used as a separate application in cream or drops; b) SL and AM can be used in addition of each other, by choosing appropriate factor from the tissue.

3.2 Spongy layer structure and biochemical composition

Little is known about the structure and biochemical composition of the SL.

Reports employing AM clinically/experimentally, merely acknowledged the SL as the inner most layer of the AM. However, probably due to the thinness of the layer, distinction from the AM, and its fragile nature and disaggregation during AM manipulation, the layer is not generally considered significant. No attempts have been made to preserve SL integrity or remove the SL from the AM, prior to use. Other than a recent publication [78] only one other report acknowledges SL during AM preparation. Yang et al 2007 [79] cites the removal of the SL using a scraping methodology, prior to preservation.

The current knowledgebase of the SL has been established indirectly through work carried out to assess the structural composition of the foetal membrane. This work was predominantly carried out in the 1990's by research groups investigating the Premature Rupture of the AM (PROM).

The structure of the SL is reported to be composed of Collagen types I, II, III, IV, V, VII and VIII [57, 183] with collagen type I and II well represented throughout [75, 76]. Collagen fibres are loosely packed and contain collagen types V and VI [183]. In addition to dense spots of collagen type IV [57, 183] other basement membrane (BM) components such as type VII collagen (anchoring plaques/rivets) [76], fibronectin, laminin, merosin and nidogen have been reported to be expressed in SL [57, 75-77]. However no fibrillin-containing microfibrils have been demonstrated[197], suggesting that SL provides no elasticity to the overall foetal membrane.

The SL contains high levels of hyaluronan [177], a major carbohydrate component of the ECM that is known to provide mechanical support and to interact with different growth factors, such as fibroblast growth factor (FGF), epidermal growth factors (EGF) and transforming growth factor (TGF-ß) [70]. The distribution of proteoglycans, collagens, and hyaluronan in SL and AM may explain the biomechanical properties of the tissues. It been suggested, that

changes in the relative proportions of ECM molecules are crucial for the proposed maturation process in the foetal membranes during the final stages of pregnancy [28].

Cells reported within the SL have been shown to express and synthesize stromelysin-1 [57] an enzyme that degrades BM components and structural components such as proteoglycans, laminin and elastin. Activation of interstitial collagenase (MMP-1) to digest fibrillar collagen and gelatinase (MMP-9) activation both require stromelysin-1 proteolysis activation [57].

According to the findings of Malak and Bell et al[237], AM or foetal membranes change their biochemical properties prior to delivery, causing structural weakness, swelling and thinning of the cellular layer.

3.2 Material and methods

With a view to assess the effect of SL, for the start, I prepared and compared three samples; 1) AM with SL intact, 2) AM with SL removed and 3) SL itself. These samples have been assessed experimentally. Depending on the effects of the samples it will enable us to determine whether to remove or leave SL intact during AM preparation and how this may be beneficial in certain articular pathological conditions. Biochemical composition of AM is vast, which give the opportunity to think about possibility of similar biochemical compositions in SL. Using a non-standardised membrane with variable amounts SL retained on the AM has several clear disadvantages:

- firstly, from a clinical standpoint, is the direct mechanical interference caused by the residual SL. In cases where the AM is transplanted SL towards the ocular surface, the residual SL is situated between the surface of the ocular surface and the AM stroma. From our observations, when the SL swells, over time, with water (discussed above), resulting in the AM being pushed away from the ocular surface (Figure 19)

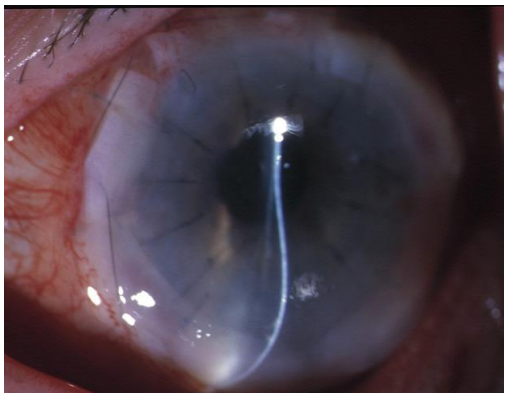


Figure 19 Slit lamp image of ocular surface after AMT.
Image of slit lamp examination of ocular surface after AM transplantation (AMT) show distortion of slit lamp light due to swelling of SL between AM and ocular surface.

- secondly, the biochemical composition of the SL is relatively unknown. To give one example, we have recently demonstrated that the SL contains high levels of TGFβ1 [111]. Furthermore, TGFβ1 is retained in SL even after preservation; therefore an inconsistent membrane retaining variable amounts of SL will also

contain variable TGFβ levels and other factors. This could have inevitable but unknown variable clinical consequences.

- furthermore, from the literature review, AMT is not 100% successful, so we can therefore assume that SL may have a role in the failure of AMT treatment in some situations. It is easy to understand why the SL is cytotoxic to cells, as embryologically this layer is a barrier between mother and baby and plays a role in immune regulation.

3.2.1 AM sample collection and preparation

The consent for the collection and processing of, placental tissue and associated membranes (amniotic sac) was carried out with agreement from the department of Fetal Maternal Medicine (FMM), QMC, University Hospitals NHS Trust, Nottingham and following ethical approval, obtained from the Nottingham Research Ethics Committee (ethics number: OY110101). The study complied with the tenets of the Declaration of Helsinki.

Consent was sought from near term patients imminently undergoing elective caesarean section. Only healthy volunteers were consented for AM donation, smokers and patients with internal diseases such as diabetes, and other endocrinological pathologies and patients with antenatal complications were excluded from this research

3.2.1.1 AM sample collection and processing

In accordance with departmental protocol, designed by Hopkinson [111], placental tissue and associated membranes were collected promptly and taken in a sterile bag from theatre after delivery. Samples were prepared under sterile conditions in a Class II safety cabinet (Envair Limited, Lancashire, England). The fetal reflectum sac was separated from the placenta by cutting around the edge of the placenta. The placenta was discarded. The isolated amniotic sac washed briefly in a sterile flask in sterile physiological saline (0.9% w/v 300ml NaCl in H_2O) (Baxter Helthcare corporation, USA) without mechanical rubbing,

to remove residual blood. The AM was then separated from the chorion using blunt mechanical removal and the isolated AM and chorion sacs washed in separate sterile T125cm^2 tissue culture flasks in sterile physiological saline 300ml for 20 minutes on a 3D rocking platform (model STR 9), at 40 rpm. The wash step was repeated three times. After washing, the AM was spread out on a sterile tray, spongy layer side up. Once the SL was removed, any attached blood clots were removed from the SL with sterile anatomical (blunt-nosed thumb forceps with serrated tips for increased grip) type of forceps. Separate AM samples, chorion samples and SL sample collected been stored in sterile 20ml tube at -80˚C, till 12 different SLs collected from different donors and pooled together for further experiments. For the first Search Light assay AM, chorion and SL samples were sent to investigate protein profile and see difference in samples proteins.

3.2.2. Spongy layer isolation and preparation

Spongy layer from 12 donors were removed according to a previously optimised protocol developed in the department [111]. Throughout the washing of AM the SL absorbed the saline solution and became swollen. A fully hydrated SL facilitates its removal from the AM. After washing, to remove the SL the AM was placed SL side up and using the reverse edge of a size 22 blade the SL was gently peeled (detached) from the AM, without unnecessary scratching or rubbing that could damage the surface of the AM and SL.

All collected SL samples were stored in universal tubes separately from each amnion collected. Only those SL samples, which prepared to be freeze dried were placed into a labelled 5 ml glass bottles. SL samples for protein extraction and cell culturing were placed in universal tubes and stored at -80°C until required for further experimentation.

3.2.3 Protein extraction

The AM, Ch (Chorion) and SL (spongy layer) samples from 12 donors were collected. Where necessary, respective AM, Ch and SL samples were pooled. Samples were weighed and subsequently ground under liquid nitrogen (LN2) using a pestle and mortar. For proteomic analysis, samples were solubilised in a Tris buffered saline with 0.1% v/v Tween containing solution, whilst for antimicrobial work samples were solubilised in TBST a powerful solubilising for the extraction of cellular membranes and structural proteins [238] (Sigma-Aldrich Company Ltd, Dorset, UK) based solution before exchanging with purified water using 3000 kDa vivaspin columns (Vivascience, Vivaspin).

Ground material was solubilised in 5 ml per 1 g 1 x Tris buffered saline with 0.1% v/v Tween and 20/Triton-X-100 µml (TBSTx, Sigma-Aldrich,) buffered for 20 min at room temperature with regular vortexing. Insoluble proteins were removed by centrifugation at 20,000 χ g, for 15 minutes, at 4°C. The supernatant was decanted off and insoluble and soluble material form each samples retained for further analysis.

3.2.4 Sample lyophilisation (freeze drying)

Whole SL, fresh AM and fresh Chorion samples were collected, washed, wet weighted, liquid nitrogen grinded, and placed in 5 ml in volume were sealed in 10 ml glass bottles and frozen at -80°C for 24 hours, to allow samples freeze fully. No medium or buffer were added to the samples. Drying was performed using an Alpha 1-4 LSC freeze-dryer (Christ, Germany). Prior to sample drying the ice condenser was equilibrated to -45°C. This allows any vapour present in the drying chamber to be removed during the drying procedure, by freezing to the condenser itself. The drying cycle comprised of a main dry phase for 24 hours (shelf temperature 15°C, vacuum pressure 1.030 mbar, safety pressure 1.650 mbar) followed by a final drying phase for 30 minutes (shelf temperature 20°C, vacuum pressure 0.0010 mbar, safety pressure 1.650 mbar). Following

completion of the drying cycle the bottles were sealed and stored at room temperature, and away from direct light, until further analysis.

Lyophilised samples were re-suspended in TBSTx (Sigma-Aldrich). A volume of 1ml TBSTx (Sigma-Aldrich) was added to each 1mg of sample. Samples were vortexed thoroughly and placed on a rocking platform for 20 minutes. Samples were then centrifuged at 2000 rpm for 2 minutes and the supernatant (5ml) was collected. This step was repeated x3 for each sample preparation with a volume of 5 ml TBSTx (Sigma-Aldrich). The proteins concentration of each sample was measured using a Bradford assay see Table 5 and the washes containing protein were combined and concentrated using 20 ml Vivaspin 3000 MWCO columns (Vivascience, Vivaspin)

3.2.8 Bradford (Coomassie staining) assay

The Bradford assay is a colorimetric protein assay, based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acidic conditions, the dye is most stable as a doubly-protonated red form. Upon binding to protein, however it is most stable as an unprotonated, blue form. Amine groups to proximity of the negative charge of dye, and the bond is further strengthened by the ionic interaction between two. Binding of the protein stabilized the blue form of Coomassie dye, and the amount of the complex present in the solution is a measure of the protein concentration by use of absorbance reading. The absorbance shift in the dye Coomassie (also known as in sodium dodecyl sulphate and blue native polyacrylamide gel electrophoresis (SDS-PAGE and BN-PAGE). The red forms Coomassie reagent changed and stabilized into Coomassie blue by the binding of protein. Two types of bond interaction take place here, the red form of Coomassie dye first donates its free proton to the ionisable groups on protein's native state, and consequently exposes its hydrophobic pockets. The exposed hydrophobic pockets on the protein chain will bind non-covalently to the non-polar region of

the dye van der Waals forces and will position the positive. The protein – dye complex causes a shift in the absorption max of the dye from 465 to 595nm. The amount of absorption is proportional to the total quality of proteins present. The linear concentration range is 0.1-1.4 mg/ml of protein.

Red (470nm) <=> Green (650nm) <=> Blue (590nm) <=> Blue-Protein (590nm)

The Bradford reagent (Sigma Aldrich, UK) was gently mixed and allowed to reach room temperature. Protein standards were prepared Table 4 using a 2 mg/ml BSA (Sigma Aldrich,UK) stock. Samples were diluted in TBSTx in the proportions given below in table 5 Bradford assay standards.

Tube number	***BSA mg/ml (µg/µ***	***Amount of stock/ml***	***TBSTx***	***Total Volume***	***Volume remains***
1	1.5	1000µl	333µ	1333µ	667µ
2	1.0	666µl	334µ	1000µ	500µ
3	0.5	500µl	500µ	1000µ	500µ
4	0.25	125µl	500µ	500µ	1000µ
5	0	0µl	1000µ	1000µ	1000µ

Table 4 Bradford assay standards.
Table represent how standards for Bradford assay were prepared in volume and concentration.

5µl of prepared standards were added, in duplicate, to separate wells in the 96 well plates. Blank wells with no protein standard contained only 5µl buffer (TBSTx) and Bradford reagent. 5µl of SL samples were added to separate wells in duplicate. To the standards and the SL samples 250µl of Bradford reagent added and samples well mixed. After incubating samples in the room temperature for 30 minutes, the absorbance was measured. The recommended wavelength was 595nm, however, the plate reader available could only read at 650nm. The readings were obtained using the L1-L2 (650nm- 405nm) measurement. Using the standard curve, the net absorbance of each SL sample was plotted and the protein concentration determined.

3.2.9 2D protein quantitation

The proteins concentration were identified by using a 2D Quant kit (GE Healthcare, UK). This method more sensitive that Commassie staining, allowed

to 1ng of proteins to be detected, however, takes more time in comparison with Bradford assay. The kit based on protein precipitation followed on copper quantification. The absorbance of the assay solution decreases, when protein concentration increasing. Assay was done according the standard protocol developed by the manufacture. Standards were prepared in 6 tubes, numbered 1-6, volume of Bovine Serum (BSA) in concentration 2mg/ml increased by 5µl for each tube (0µl-25µl) and protein quantity increased by 10µg in each tube (0µg-50µg) accordingly, so in tube 1 was 0µl of BSA and 0µg of proteins, in tube 6 25µl of BSA and 50µg proteins. At the same time colour reagent prepared by mixing two colour reagents (A and B) from the kit in ratio 100:1 – 100 part of A and 1 part of B.

In each tube 500µl of precipitant and the same amount of co-precipitant were added, samples were vortexed briefly and incubated for 3 min at RT, after each reagent added. Precipitation of the samples done by centrifugation at 15,000g for 5 min, after, supernatant carefully collected by pipetting. Pellet left in the tubes, was briefly centrifuged to separate any remaining liquid. After all visible liquid removed, 500µl of copper solution added into each tube (sample) - copper solution from kit supplied 1:4 and quickly vortexed to solubilise the pellet. Colour mix (1ml) added into each sample and vortexed well. Samples left at RT for 20 min. Protein absorbance measured on plate reader at 480nm, results compared against standard curve. As a standard for concentration samples was a minimum require for proteins in a sample 0.75mg/ml.

3.2.5 Sample preparation for Searchlight protein array analysis

During sample preparation, volume of samples was high; as three washes of each sample were perform. Samples were concentrated down using Vivaspin columns with membrane size of 3 000kDa (GE Healthcare, UK) and centrifuge with 4C to allow liquid to go through and hold proteins in the sample,

temperature of 4C standard temperature for proteins survive during sample processing and preparation without denaturation. After samples were concentrated to volume of 5 ml, protein quantitation been identified using Bradford assay to minimum (standard) amount of proteins in each sample to reach 0.75mg/ml level. Samples where amount of proteins was higher, been diluted to the “standard” protein quantitation, if protein level was low, samples were concentrated further.

A sample volume of 500μl was placed into a 1ml eppendorf and sent on dry ice for protein arrays to SearchLight immunoassay technology (Aushon Biosystems, USA). The assay were performed for 48 proteins in duplicate for each sample, data were normalised according to ng/mg of protein extract. All the rest of the samples were used for the further experiments in the laboratory.

3.2.6 Analysis Search Light results

Protein arrays were carried out by SearchLight immunoassay technology (Aushon Biosystems, USA). 48 proteins were analysed and data value normalised according to ng/mg of protein extract.

3.3 Results

All obtained data from Search Light (Table 6-15) ("Original, from SearchLight pg/ml") was calculated to the original weight of the samples and standardise to original volume ("Original Volume") (Table 6), according this figures and concentration ("Dilution factor") (Table 6) in sent samples (diluted samples while washing procedure). As dilution factor known, calculation of proteins in each sample sent for analysing were. Original figures from SearchLight assay were amount of proteins in whole sent sample in pg/ml (Table 6-15). Accordingly calculated amount of proteins in pg/ml of concentrated and undiluted SL, which transferred to total proteins in ng/mg and normalised pg/mg against concentrated proteins diluted. Using Excel software from Microsoft Office 2011 did all calculations.

3.2.7 Optimisation and validation and SL fraction separation

The SL is a very dense and gelatinous layer, which was difficult to separate into different fraction. First SL separations were done in simplest way by separating into three parts;

i) whole SL grinded, not washed;

ii) soluble SL (sSL) pellet, which is left in the tube after TBST wash;

iii) insoluble SL (iSL) concentrated supernatant (TBST with washed proteins)

To separate the maximum amount of proteins from SL, all SL samples were washed three times in TBSTx at ration of 1:10v/v. Resulting supernatant was concentrated down using 3000kDa Vivaspin columns (GE Healthcare, UK) to prepare a fine powder using LN2 and washed further with TBST. The supernatant was then collected and concentrated using Vivaspin columns. This method was adopted throughout this research project to prepare all SL samples for testing and to avoid sample variation attributed to sample processing.

The levels of proteins were measured using a Bradford assay (described above). SL samples were prepared from a single amniotic membrane and tested. However sample variations were observed and SL samples were pooled from 12 different amnion samples to eliminate any variation.

3.2.10 Mass spectrometry

Mass spectrometry (MS) identified a proteins in SL fraction (5000 – 10000kDa). Fractions were separated further to explore their biochemical composition and functions. SL extract been separated according to protein molecular weight. The MS analysis had been done by Dr Susan Liddell in the University of Nottingham. After trypsin difection, analysis perfume as a MS/MS or Tandem MS. The MS was operated with a capillary voltage of 3000 V in positive ion mode, using argon as the collision gas. Tandem MS data were acquired using an automated data-

dependent switching between MS and MS/MS scanning based upon ion intensity, mass and charge state. Protein LynxGlobalServier version 2.0 (Walters Ltd.) was used to process the MS data files were searching against all entries in Swissport and NCBInr database using the web version of the MASCOT MS/MS (http://www.matrixscience.com/).

3.2.11 Statistical analysis

Statistical analyses were performed using the nonparametric Mann-Whitney U test and $p<0.05$ was considered significant.

3.3.1 Searchlight results.

The Searchlight™ protein array analysis was carried out using a multiplex microplate-based ELISA assay and shows equivalent accuracy and superior sensitivity compared to commercially available ELISA kits [239-241]. Protein array were carried out in triplicate on biological samples from Fresh AM, TRAM, chorion, SL and placenta using Searchlight immunoassay technology for a profile of 48 protein analyses (Table 17)

The normalised levels for each protein factor were compared between placentas, chorion, SL, fresh AM and TRAM samples. 4 proteins, hECadherin, MMP1, MMP3 and IFNα were not detected in the samples. This may be due to the protein levels being outside the detection limits of the searchlight assay. All detected proteins were divided into groups according to the protein function, such as Angiogenesis[242], Biomarkers, Cell adhesion[243], Chemokine[244], Cytokine[245], Growth factors[246], Metalloprotease[247] and Neurotrophic factors.

A low number of markers (OPG, PSelectin, EGF, NT3 and BDNF) were detected at higher levels in TRAM compare to SL and only (NT3, BNGF, BDBF, TGFB1, TGFB2, l-Selectin, VCAM1) higher level in TRAM compare to fresh AM. This can be an explained as significant loss of proteins during the TRAM sample preparation (Table 17).

3.3.2 Angiogenic factors.

Angiogenesis is abnormal vessel growth that is observed in a number of ocular pathologies. Vascular endothelial growth factor (VEGF) and isoforms are the key promoters of angiogenesis. VEGF has been identified as one of the key molecules responsible for endothelial cell migration and tubulogenesis during retinal angiogenesis [248, 249] [242]. From our research, VEGF level in TRAM in 2.14 (20.91(TRAM)/9.77(SL) = 2.14) times lower compare to SL, or 46.7% in TRAM from the level of SL if to take it as 100%. Searchlight results show angiogenic factors are significantly ($p<0.05$) higher in SL than in TRAM.

FGFs stimulate growth, survival, and/or differentiation of a number of mesenchyme-derived cells and neurons. FGFs stimulate endothelial cells proliferation, migration, tubulogenesis and also angiogenesis. FGFs and their receptors are widely expressed in the eye and participate in lens differentiation, photoreceptor survival, or retinal pigment epithelium (RPE) function and survival. However the contribution of FGFs in normal and pathological vascular development in the eye have recently been elucidated [250]. The amount of FGF in Fresh AM is 100% (59.60 Table 18) the same factor in SL is 59.7% (= 35.57 (Table 18) * 100 / 59.60 (FGF Table 18)) and 4.9% in TRAM (2.92 (Table 18 FGF * 100 / 59.60 (Table 18 FGF) FGF doesn't appear to be soluble as high levels are still detected in the material after washing, unlike the loss observed between fresh AM and TRAM. The low level of FGF in chorion suggests FGF is AM derived and therefore factor detected in the SL is from the AM.

The Hepatocyte growth factor (HGF) is secreted by mesenchymal cells and targets epithelial, endothelial and haemopoietic progenitor cells. HGF has an important role in embryonic organ development, especially myogenesis. HGF levels were elevated in TRAM (818.83 (HGF in TRAM) * 100 / 4808.70 (HGF in Fresh AM – as taken for 100%) 17.03% compared to SL 65.7% (3160.95 (HGF in SL * 100 / 4808.70 (HGF in Fresh AM = 100%) (Table 17).

TIMP2 (Tissue Inhibitor of Metalloproteinase) is a member of the TIMP family is a natural inhibitor of the matrix metalloproteinase, a group of peptidases involved in degradation of the extracellular matrix. Also this protein play role against metalloproteinase, the TIMP2 has its ability to directly suppress the proliferation of endothelial cells. TIMP2 60.5% (102.67(TIMP2 in TRAM) *100/169.64(TIMP2 in Fresh AM) in TRAM (Table17) compare to SL 89.7% (152.22 (TIMP2 in SL)*100 / 169.64 (TIMP2 in Fresh AM) and Fresh AM 100%

3.3.3 Biomarkers.

Biomarkers Prolactin, PEDF and Fibronectin detected in SL sample in higher amount than in fresh AM or TRAM. Moreover, Fibronectin in SL in 33 times higher than in TRAM and in 9 times higher in fresh AM compare to SL. All other biomarkers greater concentration in fresh AM compare to SL or TRAM. First wash of AM detected very high amount of Leptin, which shows great amount of this protein washing out from the tissue.

A biomarker is a substance used as an indicator of a biological state. More specifically, a biomarker indicates a change in expression or state of a protein that correlates with the risk or progression of a disease, or with the susceptibility of the disease to a given treatment. All of the biomarkers tested were significantly elevated in SL compare with levels in fresh AM.

FASL TNF family kills T cells and activates B cells leading to down regulation of an immune response [251]. Pigment epithelium-derived factor (PEDF) also known as (SERPINF1) 24.6% in TRAM multifunctional secreted protein with anti-angiogenic, anti-tumorigenic and neurotrophic functions. PEDF inhibits endothelial cells migration and suppresses retinal neovascularization and endothelial cells proliferation PEDF is also thought to play a clinical role in choroidal neovascularization, cardiovascular disease, diabetes, diabetic macular oedema, osteogenesis imperfecta and cancer [252, 253]. As an antiangiogenic protein, PEDF may help suppress unwanted neovascularisation of the eye. Molecules that shift the balance towards PEDF and away from VEGF may prove useful tools in both choroidal neovascularisation and preventing cancer metastasis formation [254-256]. Human TNF-related apoptosis-inducing ligand (Apo2 ligand) (TRAIL ratio 1:5.4) (Table17) induces two different signals, cell death mediated by caspases and gene induced death mediated by NFkappaB.

3.3.4 Cell adhesion

Cell adhesion factors, shows interesting results, such as, ICAM1 level of (43% (187.59/80.02(Table 20)) detected in TRAM and 50% (187.59/93.09(Table 20)) in fresh AM, and VCAM1 29% (344.60/98.90(Table 20)) in fresh AM and 30% (344.60/104.30(Table 20)) in TRAM compare to level of the same factors in SL, which two times higher in ICAM 1 and three time higher in VCAM 1 (Table17) in comparison with fresh AM and TRAM. However, PSelectin level three times lower level in SL compare to fresh AM and similar level with TRAM. LSelectin in TRAM, SL and fresh AM in similar level. Those proteins are binding to the cells surface. Cellular adhesion can link the cytoplasm of the cells and can be involved in signal transduction. Cell adhesion receptors are transmembrane glycoproteins that meditate binding to extracellular matrix molecules determine the specificity of cell-cell or cell-ECM interaction [257] [257] [38]. ICAM1 expressed in amnion in preterm labour, and it is direct role not immediately obvious. However, ICAM1 expressed by the amnion would be expected during infection-associated preterm labour [258].Amount of cell adhesion proteins in 10 tested samples. Figures represent amount of proteins in ng/mg.

The cell adhesion proteins ICAM 1, ICAM 3, VCAM, hE-selectin, were significantly higher in SL compared to fresh AM.

Intercellular Adhesion Molecule 1 ICAM1 (CD54 - Cluster of Differentiation 54) is expressed by endothelial cells and cells of the immune system. The protein encoded by this gene is a type of intercellular adhesion molecule continuously present in low concentrations in the membranes of leukocytes and endothelial cells. ICAM1 can be induced by interleukin-1 (IL1) and tumour necrosis factor (TNF) and is expressed by the vascular endothelium, macrophages, and lymphocytes. ICAM1 is a ligand for LFA1 (integrin), a receptor found on leukocytes. When activated, leukocytes bind to endothelial cells via ICAM1/LFA1 and then transmigrate into tissue [259, 260].

ICAM3 (CD50) is constitutively and abundantly expressed by all leucocytes and may be the most important ligand for LFA1 in the initiation of the immune response. It functions only as an adhesion molecule, but also as a protein-signalling molecule [261, 262].

VCAM1 (CD106) is expressed by blood vessels following cytokine stimulation of endothelial cells. The VCAM1 protein mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to the vascular endothelium. It also functions in leukocyte-endothelial cell signal transduction, and it may play a role in the development of atherosclerosis and rheumatoid arthritis.

E-Selectin (CD62E antigen-like family member E, or ELAM1 - endothelial-leukocyte adhesion molecule 1 or LECAM 2 – leukocyte-endothelial cell adhesion molecule 2, is a cell adhesion molecule expressed by endothelial cells. During inflammation, E-Selectin plays an important part in recruiting leukocytes to sites of injury. E-Selectin mediates the adhesion of tumour cells to endothelial cells, by binding to corresponding ligands expressed by neutrophils, monocytes, eosinophils, memory-effector T-like lymphocytes, natural killer cells or cancer cells.

3.3.5 Cytokines

Twelve cytokine proteins tested, some proteins like IL7, IL3, were not detected in the TRAM, fresh AM and SL Amount of IL5 in SL double compare to fresh AM and TRAM, where this protein in similar amount. TNFα not detected in the TRAM, however, in SL much low then in fresh AM (nearly 4 times). IL2 in fresh AM in much greater concentration then in TRAM and SL. IFNg lowest in TRAM, only 2.5% (0.00924*100/0.3653 (Table 21)) compare with SL and fresh AM, where this protein in similar amount. IL1b, IL4, IL10 in all three compared samples have similar amount. Interestingly, IL1ra in fresh AM in double amount compare to SL, but four times lower in TRAM compare to fresh AM (Table17).

Cytokines can be separated according to their properties. Pro-inflammatory cytokines include IL-1β, TNFα, IL-1α and IL-8 [189, 243, 263] [264]

The main functions of IL-1β and IL-1α are to promote activation, co-stimulation and secretion of cytokines and other active-phase proteins.

IL-8 (CXCL8) is a chemokine also known as neutrophil chemotactic factor. It is secreted by a variety of cell types including monocytes and macrophages, T-cells, neutrophils, fibroblasts, endothelial cells and tumour cells in response to inflammatory stimuli. Levels of IL-8 correlate with histological grade in glial neoplasms and the most malignant form glioblastoma shows the highest expression in pseudopalisading cells around necrosis, suggesting that hypoxia/anoxia may stimulate expression [265]. Accumulating evidence has demonstrated that various types of cells can produce a large amount of IL-8 in response to a wide variety of stimuli, including proinflammatory cytokines, microbes and their products, and environmental changes such as hypoxia, reperfusion, and hyperoxia. Many observations have established IL-8 as a key mediator in neutrophil-mediated acute inflammation due to its potent actions on neutrophils. However, there are evidence indicate that IL-8 has a wide range of actions on various types of cells, including lymphocytes, monocytes, endothelial cells and fibroblasts, besides neutrophils. Those all evidence suggest that IL-8 has a crucial role in various pathological conditions such as chronic inflammation and cancer. IL-8 has been associated with tumour angiogenesis, metastasis, and poor prognosis in breast cancer. IL-8 may present a novel therapeutic target for estrogen driven breast carcinogenesis and tumour progression [263, 266-269].

The main function of TNF-α is inflammatory, promoting the activation and production of acute-phase proteins.

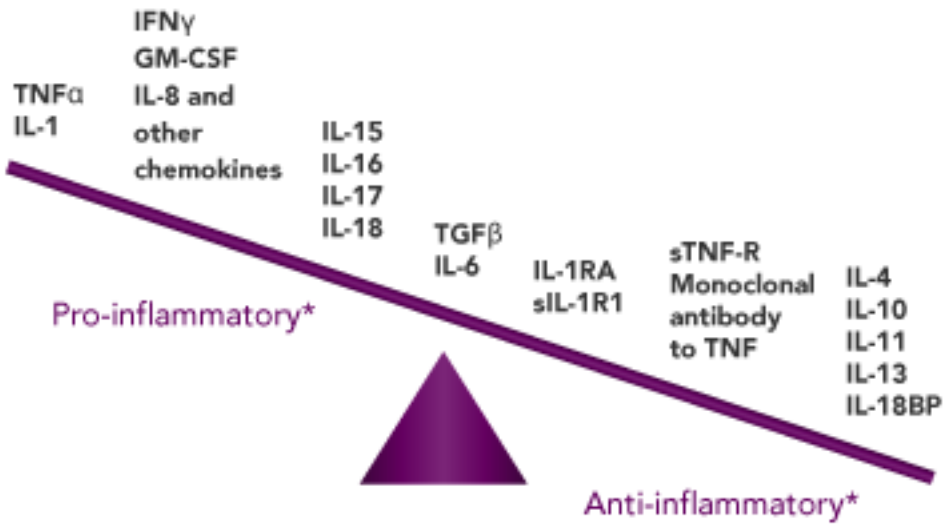
All pro-inflammatory factors [189, 270] were detected in SL and were higher compared to the levels measured in FRAM.

Anti-inflammatory cytokines include (IFNγ, IL-10, IL-6, and IL-1ra).

Looking at the panel of anti-inflammatory factors measured only IL-6 was significantly elevated in SL compared to FRAM. No statistical changes were observed with IL-10, IL-1ra and IFNγ Table21.

IL-1ra and the soluble decoy receptor complex inhibit IL-1 mediated inflammatory responses. IL-6 inflammatory and co-stimulatory action induces proliferation and differentiation and synergizes with both TGF-β to drive T helper 17 cells.

IL-10 is involved in immune suppression, decreasing antigen presentation and major histocompatibility complex (MHC) class II expression of dendritic cells, down-regulates pathogenic Th1, Th2, and Th17 responses. IFNγ promotes activation of antigen presenting cells (APCs) and cell-mediated immunity by increasing MHC class II expression.



Cytokines drive the inflammatory response[5]

Figure 20 Cytokines drive the inflammatory response. Figure shows the cytokines with inflammatory response. This image belongs to:
http://www.jakpathways.com/cytokines?loc=eu&source=google&HBX_PK=s_cytokines&skwid=43100000700000001

3.3.6 Growth factors

Overall, SL samples have a higher concentration of growth factors compared to TRAM and fresh AM except EGF and PLGF. The EGF and the PLGF only two factors in the group, where amount of detected factor in SL less than in the TRAM and in fresh AM. In SL protein detection was comparable to fresh AM 32% (3.5/10.8) and TRAM 69% (3.5/5.1) for EGF (Table17). For PLGF (Table17) TRAM and fresh AM 25% (0.013/0.052) compare to SL. Greatest difference in TGFβ2 (Table17) fresh AM 42.6% (8.66/20.30) compare to SL and 57% (11.62/20.30) TRAM compare to SL and HGH (Table17) TRAM 46% (3.79/8.33) fresh AM 56% (4.63/8.33) compare to SL.

Epithelial growth factor (EGF) and basic epidermal growth factor (BEGF) belong to the same family [271] of proteins and stimulate cell growth, proliferation and differentiation by binding to its receptor, epidermal growth factor receptor (EGFR). Similar levels of expression of both EGF and BEGF were observed in SL and FRAM. However, HGH, SCF, TGFβ1 and TGFβ2 expression is significantly higher in SL compare to FRAM.

Human Growth Hormone (HGH) stimulates growth and cell reproduction in humans and other animals. Stem cells factor (SCF) plays an important role in haematopoiesis, spermatogenesis and melanogenesis.

Transforming growth factors-beta (TGFβ) polypeptides are involved in the regulation of cellular processes, including cell division, differentiation, motility, and adhesion and cell death. The TGF-β1 inhibits the secretion and activity of some cytokines including interferon-γ, TNF-α and some interleukins. Additionally TNF-α can decrease the expression levels of certain cytokine receptors but may also increase the expression of certain cytokines in T cells and promote their proliferation, particularly in immature cells. TGF-β1 also inhibits proliferation and stimulates apoptosis of B cells, and plays a role in controlling the expression of antibodies. TGF-β1 plays an important role in bone remodelling, as it is a potent

stimulator of osteoblastic bone formation, causing chemotaxis, proliferation and differentiation in committed osteoblasts. Once cells lose their sensitivity to TGF-β1-mediated growth inhibition, autocrine TGF-β signalling can promote tumorogenesis. Elevated levels of TGF-β1 are often observed in advanced carcinomas, and have been correlated with tumour invasiveness and disease progression [272-275].

TGF-β2 regulates key mechanisms of tumour development, such as immunosuppression, metastasis, angiogenesis and proliferation. The signalling pathway of TGF-β2/Smad plays an important role in the pathological process in posterior capsule opacification following cataract surgery. Silencing Smad2 ((Mothers against decapentaplegic homolog 2) mediates the signalling of TGF-β, and therefore regulates multiple cellular processes, such as proliferation, apoptosis and differentiation [276, 277]). Smad3 ((Mothers against decapentaplegic homolog 3) is involved in cell signalling and it modulates activin signalling and [268, 278-280] efficiently blocks the effect of TGF-β2 on cell proliferation, migration and extracellular matrix production. In addition, the upregulation TGF-β2 level is a common pathological feature of Alzheimer disease and suggests that it may be closely linked to the development of neuronal death related to this disease [281-284].

3.3.7 Metalloproteinases

Level of Metalloproteinases TIMP1 (Table17) 80% (270.71/217.15) compare SL with fresh AM and 62% (270.71/167.07) in comparison SL with TRAM, MMP2 (Table17) 24% (874.02/209.12) for TRAM and 45% (874.02/389.93) for fresh AM, and MMP8 (Table17) 5% (22.12/1.04) TRAM and 27% (22.12/6.05) fresh AM in SL higher than in fresh AM and TRAM. MMP1 and MMP3 were tested as well, but were not detected in the samples (Table17).

3.3.8 Neurotrophic factors

Level of neurotrophic molecules in SL sample very similar to the same factors in fresh AM and TRAM, however, only CNTF significantly higher in SL (Table 17 and Table 24) compare to TRAM 24.4% (4.0*100/16.42) and fresh AM 66% (10.77*100/16.42). Neurotrophic factor genes are expressed in AM and choriodecidual membrane, some of them are increased with labour at term or in association with infection preterm, which suggest role of these in the process of labour [285].3.3.9 2D - electrophoresis

The result of two dimensional electrophoresis (2DE), shows presentation of the proteins in all washes. An abundant proteome in water washed SL sample, was relatively constant across all samples of the SL. In comparison of water washed with TBST washed samples, abundant proteome were significantly low in TBST washed SL sample. Samples washed in IEF (isoelectric focusing) buffer (Ettan IPGphor3^{TM} IEF System (GE healthcare)), which known as a more powerful for structural proteins, showed more high weight protein spectrum.

Interestingly, across all different washes (water, TBST, IEF) some proteins badges appealed constantly.

Figure 21 2-D gel proteins in SL.
2-D gel shows proteins in SL

3.3.10 MS results

As searchlight identified a profile or trophic factors in SL extract, in attempt to further identify beneficial trophic factors, mass spectrometric characterisation was employed. As many growth factors are 5kDa to 10kDa, this fraction was screened initially. MS/MS identified proteins were separated into groups according their functions and localisation in the tissue. As can be seen from the Figure 22 below, majority proteins localised in cytoplasm. In Figure 23 proteins presented according their molecular function. Vast number of proteins has different binding activity, such as binding ATP (adenosine triphosphate) inhibits thrombin, neiropsin and chymotrypsin, but not trypsin (PEBP1_Human Phosphatidylethanolamine-binding protein 1 OS=Homosapiens GN=PEBP1 PE=1 SV=3); binding calcium ion (S10A4_CANFA Protein S100-A4 OS=Canis familiaris GN=S100A4 PE=3 SV=1); binds to actin and affects the structure of the cytoskeleton (PROF1_BOVIN Profilin-1 OS=Bos taurus GN=PFN1 PE=1 SV=2). Oxidoreductase activity, cell proliferation (THIO_HUMAN Thioredoxin OS=Homo sapiens GN=TXN PE=1 SV=3); muscle organ development (TAGL2_BOVIN Transgelin-2 OS=Bos taurus GN=TAGLN2 PE=2 SV=3); modulator of glutaredoxin biological activity (SH3L3_BOVIN SH3 domain-binding glutamic acid-rich-like protein 3); and keeps inactive conformation of TGFß1 (FKB1A_BOVIN Peptidyl-prolyl cis-trans isomerase FKBP1A OS=Bos) for all this four functional activity percentage is 9.

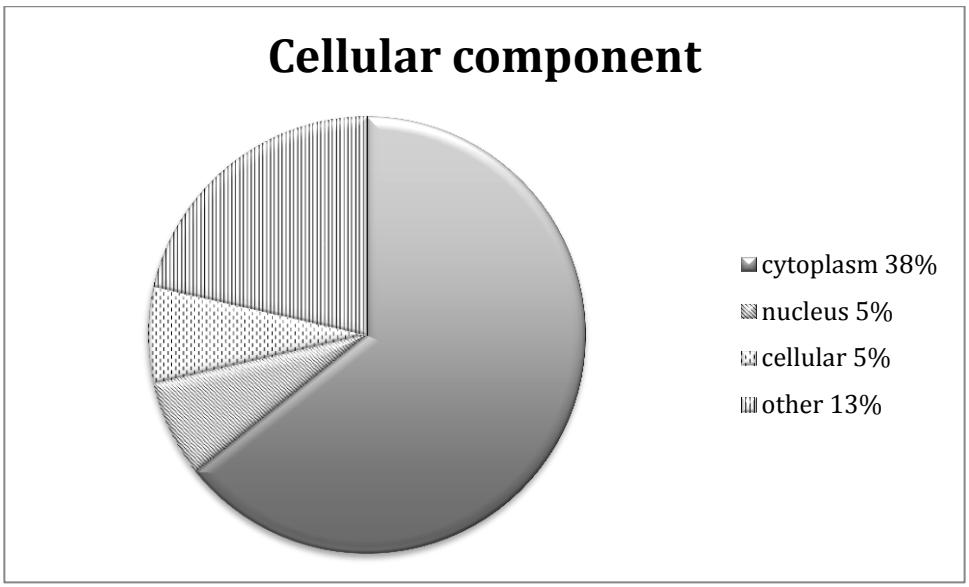


Figure 22 Distribution of proteins in SL sample according to sub-cellular location. Distribution of proteins identified in SL sample according to sub-cellular location.

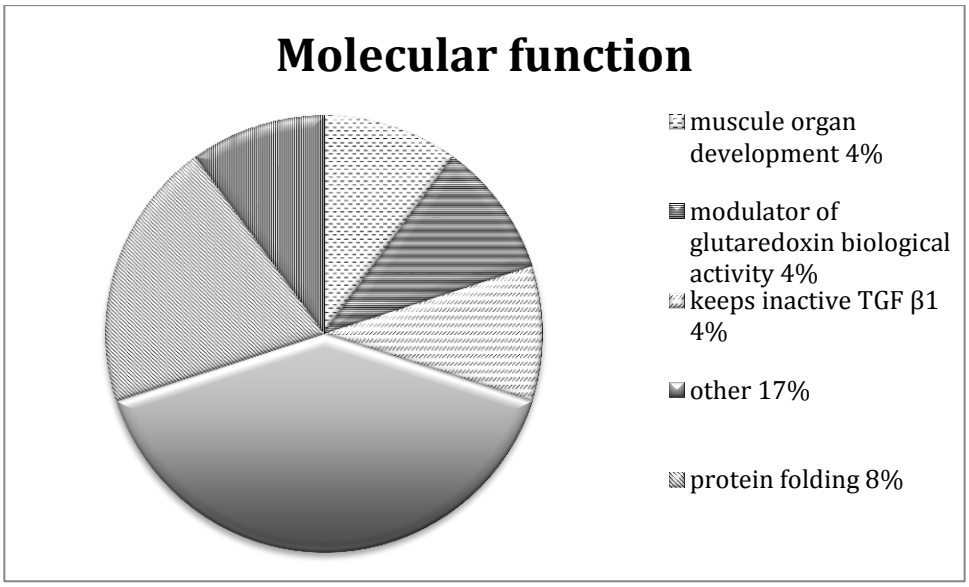


Figure 23 Distribution of proteins in SL sample according to molecular function.

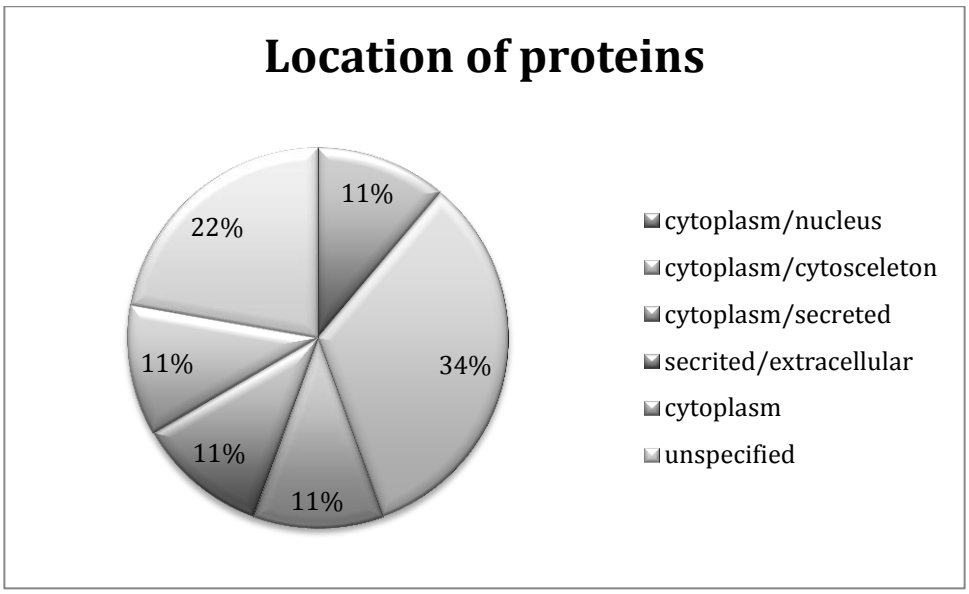


Figure 24 Location of proteins detected in SL. Location of proteins detected in SL according to the original location.

Majority (67%=11%+34%+11%+11%) proteins from SL are cytoplasm proteins migrated from the neighbour's layers. Unspecified proteins are 22% and secreted/extracellular proteins are 11%.

3.4 Discussion

The some proteins in a sample are ubiquitous, with little relevance to a particular study. Therefore, unless identifying proteins in a restricted fraction, i.e. segregate a population of specific proteins. The aim of the study was to characterise proteins in SL: therefore, proteins segregation was not an option. Grouping identified proteins according to type, function, sub-cellular location according to which other proteins they interact with, provided a snapshot of expressed proteins at the point of sampling. This was then interpreted in two ways;

1) Characterising SL activity, structure and function *in vivo*;

2) Relative to function and properties of interest.

From the first protein assay, could make a conclusion, that while TRAM preparation, some proteins in significant amount are washed from the membrane and, as proteins presented in wash, and level of those proteins are different in TRAM and fresh AM. At the same time many proteins are staining in SL in higher amount than in TRAM, some of them even in higher amount in washed SL, it means, that SL absorbing proteins while washing as well as water. This can be valuable property to keep needed proteins in SL. Difference in proteins amount varies from protein to protein.

The Angiogenesis proteins such as VEGF, IGFB1 and TIMP1 are in higher amount presented in SL. By looking at HGF, amount of this protein in 7 times higher in washed SL sample compare with unwashed SL sample, which is clearly absorption of the protein.

Biomarkers Prolactin, Fibrinonectin and PEDF in greater amount in SL than in TRAM and fresh AM, and all biomarkers insignificantly higher amount in SL washed compare with SL sample. This is important to know in process of preparation SL sample, vast majority of proteins are losing of the sample.

Cell adhesion molecules in significantly higher amount in SL sample compare with TRAM and fresh AM, which is can be important founding and have big clinical usage not just in ophthalmology, as those molecules found to contribute to the increased adherence and extravasation of LFA-1 (Lymphocyte Function-associated Antigen 1, presented on T-cells, B-cells, macrophages and neutrophils) and VLA-4 (Very Late Antigen-4, expressed on leukocyte plasma membranes) positive leukocytes in chronic venous insufficiency [286].

Cytokines IL5 and IL9 molecules in SL sample in higher concentration, then in TRAM and fresh AM. The Cytokine proteins IL1ra (10 times) IL4 (13 times) TNFα (14 times) have significant difference in comparison between washed and unwashed SL [287].

Metalloprotease MMP2, MMP8, TIMP1 molecules in SL in very high amount compare to TRAM and fresh AM, According to Malak and Bell, MMPs play role in morphologic [48, 101, 237] changes and biochemical [101, 288, 289] including increasing thickness of the connective tissue of amnion and chorion. This was found, that level of MMPs increased prior to delivery [290, 291].

In SL sample Fibronectin is in the highest amount compare to all other samples and other proteins. This is proof of mainly described characteristics of the SL as a gelatinous substrate, which one of the main functions to allow amnion slide against chorion prior to growth embryo.

Mass spec analysis shows cytoplasm proteins presented in SL sample, those proteins can be migrated proteins, as cytoplasm is a cellular gelatinous substance, however, it was proved that SL is non-cellular layer. Consequently, SL's cell presented in SL are migrated cells from AM or Chorion.

Through all tests interestingly to note, those SL proteins are easily washed from the sample with water. Which is significant founding, as AM and SL

preparation from AMT involved washes, where tissue losing the significant amount of valuable proteins.

3.4.1 Recognition of the SL

Reports employing AM clinically/experimentally, merely acknowledged the SL as the inner most layer of the AM. However, probably due to the thinness of the layer, distinction from the AM, and its fragile nature and disaggregation during AM manipulation, the layer is not generally considered significant. No attempts have been made to preserve SL integrity or remove the SL from the AM, prior to use. Other than a recent publication [78] only one other report acknowledges SL during AM preparation. Yang *et al* 2007 [79] cites the removal of the SL using a scraping methodology, prior to preservation of the AM.

To date, there is no previous ophthalmic research literature or research in other medical subspecialties describing the use of AM specifically with or without spongy layer. Tseng reference the SL on two occasions. The first was in his patent “Grafts made from amniotic membrane; methods of separating, preserving, and using such grafts in surgeries”, acknowledging the SL as part the anatomical structure of the AM [292]. However, although his patent describes the preparation of the AM, there is no mention of removing or preserving the SL. The second was in a manuscript, where it says the AM and chorion are fused and that the ‘spongy layer’ is the space generated during processing [80]. This is not accurate as the SL occupies a space between the layers [101], but may expand further during processing.

TIMP2 has a unique role among TIMP family members in its ability to directly suppress the proliferation of endothelial cells [293]. Those proteins are in higher level presented in the SL.

3.5 Summary

Due to the lack of understanding regarding the SL and it is biochemical composition further research is required to establish the clinical significance of retaining or fully removing the layer. As an alternative to removing the SL, after washing the clean membrane can be prepared directly for preservation therefore preserving the intact SL. The functions of the AM with or without SL can then therefore be assessed experimentally and clinically.

Insoluble SL (iSL) exhibits a cytotoxic effect. The apoptosis assay, showed that exposure of corneal keratofibroblasts treated with the fractions of SL which containing high molecular weight proteins do cause the cells death by necrosis rather than apoptosis. However, apoptosis occurs in those corneal keratofibroblasts, which were exposed, to fractions with lower molecular weight proteins.

Conversely, the SL removed from the AM during processing can be retained for further analysis. Our preliminary analysis of this material has identified a plethora of factors, which have future potential for clinical exploitation. However, more importantly, identification of such factors has provided us with a basis for understanding the role of the SL in ocular wound healing.

During fetal development, the immune system has to develop simultaneously as it needs to be protected against any invading pathogens that may harm the fetus. Therefore, as the fetus cannot protect itself, this protection has to come from the surrounding environment. From previous work, antimicrobial properties of AM have been demonstrated [294]. AM plays a role as a physical barrier against infection, but its mechanism still needs to be confirmed.

Potential antimicrobial properties of AM would be beneficial in the treatment of wounds. When used as a biological bandage AM acts as a physical barrier against microbes, protecting wounds from potential infection [61].

3.5.1 Potential clinical significance of the SL

Preliminary data from culture experiments, suggest adding SL to cultures kills both corneal epithelial cells (CEC) and keratocytes. However, adding SL extract to the cells promotes cell growth, increasing the rate at which cells reach confluence. If SL is demonstrated to be cytotoxic, this may have significant clinical implications, particularly as the SL is typically ignored ophthalmically, resulting in variable and partial SL removal. An initial cytotoxic effect of SL followed by a release of factors, which promote cell growth may explain in part the some of the properties attributed to the AM. However, these results are preliminary and require further validation.

Chapter 4 - Spongy Layer Fractionation

4.1 Introduction

From first Search Light results SL have vast amount of proteins, which present in SL sample in greater amount compare with TRAM and fresh AM. To identifiy those factors further SL was fractionated according to the size of proteins, by using Vivaspin columns. Insoluble Spongy Layer (iSL), see “3.2.7 Optimisation and validation and SL fraction separation”, been fractionated further for the analysis. For the second Searchlight samples were prepare to see variations in the SL samples to answer the question of how many SL from different patients need to be pooled together to reduce biological variations. Second purpose was to compare SL properties with TRAM.

Fractionated SL samples were used in all further experiments.

4.2 Methods and materials.

4.2.1 Proteins from the SL were fractioned in two different ways:

4.2.1.1 Revers Phase Solid Phase Extraction (RP-SPE) isolation of proteins

RP-SPE is based on the same principle of affinity-based separation as liquid chromatography. SPE can be in normal phase, reverse-phase and ion exchange models. The reverse-phase is more widely used [295, 296]. For this assay two types of cartridges were used: International Sorbent Technology 25 mg 1000Å PDVB (polydivinylbenzene) for protein and 50mg ENV+ 60Å PDVB for surfactant or peptide. And two solvents:

- Solvent A – water 0.1% (v/v) TFA (100ml water + 0.1ml TFA)
- Solvent B – 90% (v/v) acetonitrile 0.1% (v/v) TFA 90 ml MeCN + 10ml water +0.1ml TFA.

To desalt, proteins were placed in 60Å cartridge and Solvent A been added. For protein elution Solvent B fractionated (20% (v/v) MeCN 0.1% (v/v) TFA; 30% accordingly; 35%; 40%; 43%; 45%; 48%; 50%; 70%; 90% been used.

4.2.1.2 Soluble Protein fractionation.

These columns are designed for fast, non-denaturing concentration of biological samples by membrane ultrafiltration[297]. These columns were chosen, as they prevented samples from drying, as concentration of each portion of the samples took many hours, because gelatinous substrate had a difficulty to go through columns even in high dilution (ten times). The vertical polyethersulfone membrane minimizes membrane blockage. Each single tube contains two compartments, one upper compartment containing sample and lower compartment separated by semipermeable membrane with a specific molecular weight cut-off (MWCO). Centrifugation was used to force the solvent through the membrane, leaving a more concentrated sample in the

upper compartment. Samples were concentrated ten times; original volume was ten times higher, than final concentrated volume.

The company advised using a MWCO at least 50% smaller that the molecular size of the species of interest. This made it very difficult to separate the samples accurately.

The flow rate of Vivaspin columns is approximately 1.5 times slower at 4ºC than at 25ºC and viscous solutions take up to 5 times longer to flow through the membrane than samples in a predominantly buffer solution. SL samples are very thick, gelatinous and protein separation proved difficult. It was not possible to increase the temperature above 4ºC, without risk of denaturing the proteins.

The polyethersulfone membrane used in Vivaspin columns Figure 25 (GE Healthcare Life Sciences, USA) is extremely low protein binding minimizing the likelihood of target analyse binding [297]

Figure 25 Vivaspin column.
Shows the model of vivaspin columns which been used during the work for sample preparation.

SL was fractionated according to molecular weight at several size levels Table 5.

Sample number	***Molecular size proteins***
1	3-10.000kDa
2	10-30.000kDa
3	30-50.000kDa
4	↑ 50.000kDa

Table 5 Fractionated SL samples. Samples were sent to Aushton Biosciences (Searchlight TM, Endogen, Perbiosciences, MA, USA) for protein microarray analysis.

This size fractionation was achieved by using Vivaspin MWCO 1 000 000, Vivaspin MWCO 300 000, Vivaspin MWCO 100 000, Vivaspin MWCO 50 000, Vivaspin MWCO 30 000, Vivaspin MWCO 10 000, Vivaspin MWCO 5 000 and Vivaspin MWCO 3 000 were used. These columns were produced by GE Healthcare Life Sciences and designed for fast, nondenaturing concentration of biological samples by membrane ultrafiltration [298]. Columns designed in a way to stop samples from dryness and enables direct concentrate recovery. The vertical polyethersulfone membrane minimizes membrane blockage and tolerates high flow rates, which is a perfect solution to concentrate or purify proteins from gelatinous samples such as SL. Each single tube contains two compartments, one upper compartment containing sample and lower compartment separated by semipermeable membrane with a specific molecular weight cut-off (MWCO). Centrifugation was used to force solvent through the membrane, leaving a more concentrated sample in the upper compartment.

Vivaspin columns are fraction columns, starting with isolating the largest protein sizes, and then decreasing size. First soluble SL extracts were placed in Vivaspin column with MWCO 1,000,000 kDa and centrifuged at 15,000 rpm (or 21,380 x g) for approximately ten hours until whole sample gone through the column. The remaining pelleted material that did not pass through the column, was recovered in 2ml of the remained volume and, labelling as a fraction with proteins greater than 1 000 000 kDa in size. The collected supernatant passed through the column, was then re-centrifuged through a 300 000 kDa Vivaspin column at the same parameters until the minimum volume had been reached. Again, the remaining pellet was retained and labelled as a fraction with proteins size between 1 000 000 kDa to 300 000 kDa. This method been used till all possible columns size used. After checking

proteins in each fraction, decided to pool some fractions tougher and made only fractions Table 5 to use in all future experiments.

The premise of this research chapter was to look at SL as an independent layer and its function, properties and embryological origin in relation to amnion and chorion.

My primary focus was to assess the response of corneal epithelial cells (CEC), keratofibroblasts and human lymphocytes to SL. This was achieved by culturing the different cell types with whole SL and two fractions: soluble (sSL) and insoluble (iSL).

In parallel I tested the antimicrobial properties, cytotoxic and proliferation potential of SL fractions

4.2.2 Sample variation and preparation

iSL been separated into different fractions according to molecular weight (size) of proteins.

Sixteen samples from different donors were collected and prepared as previously described random eight samples selected as a single sample, others were grouped in 4a - first 4 samples from selected 8, 4b – second 4 samples from selected 8, 8a – all random selected 8 samples, 8b – another sat of 8 samples, and all 16 samples pooled together Table 6.

Sample number	***Original sample number according to AM number from departmental record***
1	SL 175
2	SL 179
3	SL 192
4	SL 197
5	SL 173
6	SL 178
7	SL 182
8	SL 183
9	4a Samples
10	4b Samples
11	8a Samples
12	8b Samples
13	16 Samples

Table 6 List of SL samples sent to Aushton Biosciences SL were collected from AM with departmental registration number according to the AM number and five samples were combined in 4, 8 and 16 different donors to exclude donor variations.

4.3 Results

4.3.1 Second Search Light results.

4.3.1.1 Chemokine

Chemokine proteins were tested in second Search Light assay, and found that, RANTES and MIP1β in statistically significant amount higher in SL compare to FRAM (fresh AM). Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) or Chemokine (C-C motif) ligand 5 (CCL5) is chemotactic for T-cells, eosinophils and basophils, playing an active role in recruiting leukocytes to inflammatory sites. RANTES in combination with IL2 and IFN-γ released by T cells induce the proliferation and activation of certain natural-killer (NK) cells to form CHAK (CC-Chemokine-activated killer) cells.

Together with Macrophage inflammatory Protein 1β (MIP-1β) has been identified as a natural HIV-suppressive factor secreted by activated CD8+ T cells and other immune cells [299, 300].

Those tables shows the amount of each factor, molecular weight of protein and the amount of that protein detected in each of the defined fractions. As mentioned previously, fractions were separated using this methodology for simplicity. It was not possible to separate individual proteins or groups of proteins with similar function due to natural biological properties of each protein and the dense composition of the SL.

Fraction contains molecular weight proteins less than 50 000kDa, including MIF (Macrophage migration inhibitory factor 13.8kDa – immunoregulatory cytokine, plays a particularly critical part in cell cycle regulation and therefore in tumorigenesis, MIF stimulates IL-1, IL-8 and MMP expression on fibroblasts) stand out significantly compare to other proteins. The Amount of Fibrinogen growth steadily from fraction with low molecular weight to higher. PEDF (50kDa) expressed in each fraction, however, in fraction of 30 000-50 000kDa significantly high.

From the tables, represented each protein we can see that fractions not clear, some proteins presented in different molecular weight groups, where they in theory should not be. Some of the reasons for this are the way how vivaspin columns work. Alternatively another reason may be the biological properties of proteins, which can have different shape, not many proteins have linear form, and majority have 3-D structure.

TGF-β1 (44.3kDa) [301-303] is a diametric cytokine, however, TGF-β1 can be found as a small latent complex (SLC) with a molecular mass of 25kDa [304]. Recombinant TGF-β1 is a disulphide- linked monomer protein consisting of 113 amino acids residue subunits. TGF-β1 migrates as 12-13kDa protein under reducing conditions on SDS-PAGE This may be the reason TGF-β1 is

expressed as a protein with molecular weights of approximately10kDa and greater than 50kDa [305].

PEDF has molecular weight of 50kDa, and is eluted in fraction 30-50kDa and more than 50kDa. This may be explained by PEDF protein folding to form a protein with a molecule weight of more than 100 kDa [306].

TGF-β2 with molecular weight of 47.4kDa is similar to TGF-β1. TGF-β2 forms a ring from eight amino acids that are held together by two disulphide bonds while the third one passes through the centre of the protein. The seventh conserved cysteine residue forms an intermolecular disulphide bond between two monomers to form a dimer. Both forms of TGFβ could be localized at the cell surface and this interaction has been shown to be necessary for the activation of latent TGFβ. Additional proteins are frequently associated with the SLC to yield the LLC (larger latent complex). The latent TGFβ-binding protein (LTBP) is a glycoprotein of 125-190kDa that is covalently bound to LAP (latency associated protein) through its third eight-cysteine region[307]. This may explain why TGF-β2 is present in the fraction containing molecular weight proteins greater than 50kDa.

IL1-α has a molecular weight of 30.7kDa and is predominantly expressed in fraction 3 000-10 000kDa. This can be explained by the expression of the mature form or biologically active form of the IL-1α (17kDa) [308] which presents we can see in fraction 10 000-30 000kDa.

IL-8 was mostly expressed in fraction 3 000-10 000kDa.IL-8 is 8.9kDa, and this protein can form diametric isoforms double in size which allow it to be expressed in the fraction containing protein greater than 10 000kDa. The polypeptide chain of MIP-1β contains 69 amino acids and has a molecular weight of 7.7kDa. This protein was expressed in fraction group 3 000-10

000kDa and in larger group as an attached form with other proteins, biologically active to human blood monocytes [309, 310].

RANTES (7.8kDa) was expressed in fraction group 3 000-10 000kDa, as this cytokine structurally belong to the interleukin 8 superfamily of leukocyte-selective attractants, and that is known to be a “memory-type” T lymphocyte-selective attractants. Electrospray mass spectrometry (ESP-MS) of EoCPs revealed for EoCP-2 a molecular mass of 7,862.8+/-1.1 Daltons, which is 15.8 mass units higher than the calculated value of RANTES [311, 312].

SCF is 55.9kDa but the soluble form of SCF has a molecular weight of 18.5kDa [313]. This explains why SCF is expressed in fraction group 10 000-30 000kDa. The soluble form forms a dimer, double in size and allows SCF to be detected in fraction group 30 000-50 000kDa and greater than 50 000kDa.

TSP1 is 129kDa in molecular weight and is predominantly expressed in fraction group greater than 50 000kDa. However, TSP1 protein may exist [265], in different molecular weight isoforms [314].

BDNF 27.8kDa contains about 50 amino acids identity with NGF, NT-3 and NT-4/5. Each neurotrophin consists of a noncovalently-1 linked homodimer and contains a signal peptide following the initiation codon; and a pro-region containing an N-linked glycosylation site. Initially produced as proneurotrophins, prohormone convertases such as furin cleave the proneurotrophins (MW 30kDa) to the mature neurotrophin (MW 14kDa) [315]. Neurotrophins also share a distinctive three-dimentional structure containing two pairs of antiparallel β-stands and residues in a cysteine knot motif [316].

MIF 13.8kDa this is a monomer consisting of 115 amino acids, forming a homotrimer [317]. Research [318] has shown test chains of 50-65 amino acids to have “MIF-like biological function”, suggesting that this exposed region of the protein is responsible for much of MIF’s activity and low molecular weight.

Also MIF assembles into a trimmer composed of tree identical subunits [264, 319]. This is explained why MIF is expressed in all fraction groups.

CNTF 22.8kDa, single non-glycosylated polypeptide chain containing 200 amino acids [320, 321].

Fibrinogen 340kDa is huge protein, which is present in fraction with molecular weight more than 50 000kDa.

Chapter 5 – Effect of Isolated SL and Cells Proliferation and Apoptosis

5.1 Introduction

The effect of AM on corneal epithelial cells is well known, AMT promotes the rapid epithelialisation [107, 322, 323] and culturing of epithelial cells [324]. The AMT is beneficial in the inflammatory response of the corneal surface [124, 148, 294]. The biochemical profile of the SL was investigated using the Searchlight multiplex array. The TBSTx (tris-buffered saline-Triton-X 100) soluble fraction of the SL was shown to possess significant levels of growth factors, cytokines, anti-inflammatory molecules, cell adhesion molecules and neurotrophic factors. The levels of some of the above mentioned factors found in the SL were comparable to levels found in fresh amniotic membrane. This finding, will allow us to separate two tissues and use their biological properties depending on the pathology (disseise) and the expected outcome from treatment with amniotic membrane. This point gave us the idea to test the effect of SL on CEC (corneal epithelial cells), KFB (keratofibroblasts) and human lymphocytes as movable cells. First of all, SL samples that were not fractionated were tested on all cell types. For that purpose human lymphocytes were chosen as movable cells to exclude the osmolar effect of SL and to investigate cells behaviour on SL treatment.

In the literature the effect of AMT was described as the immunosuppressive ability of AM, however, the effect of that ability is still unknown [324, 325].

5.2 Methods and Materials

5.2.1 Cell culture

5.2.1.1 Primary keratocyte isolation and culture.

Primary keratocytes (pKer) were isolated according to a previously published methodology [326]. Cornea rim (rim is a corneoscleral ring without conjunctiva, and central part, which been cut for corneal grafting) were obtained from the Manchester eye bank. The epithelial and endothelial layers were removed from residual peripheral cornea by mechanical scraping, using a No 22 blade, which was then used to mince the remaining limbal tissue. The tissue was subsequently digested in 0.1 mg/ml collagenase type IA (Sigma Aldrich, UK) and digested for approximately 18 hours at 37°C, 5% v/v CO2. After that time, the solution was filtered with a 41µm nylon filter (Fisher Scientific, UK), to remove non cellular debris. Culture medium was added to the filtrate solution prior to centrifugation at 450 x *g* for 6 minutes. The cell pellet was resuspended in FCM (serum free medium) medium (Sigma-Aldrich) supplemented with 20% v/v heat- inactivated FBS (Fetal Bovine Serum) (Fisher Scientific), 2.5 µg/ml Plasmocin™ (Autogen Bioclear, UK) 0.02 µg/ml gentamicin, 0.5 ng/ml amphotericin B (combination Gibco, Invitrogen, UK) and 1.59 mM L-glutamine (All Sigma-Aldrich).

After rim separation, stromal part of the rim, were cut into small pieces, as small as possible, to increase “working” surface, and were placed into in 0.1% collagenase Type IA (Sigma Aldrich, Dorset, UK) to DMEM medium, filters-sterilized (Minisart High-Flow, Sartorius Stedim Epsom, UK) with pore size of 0.20 µm were used. Universal tube were used for that purpose, tissue were incubated overnight 37°C, 5% CO_2, 95% humidity). After, solution with tissue was filtered using net filter (Fisher Science, Loughborough, UK) and deactivated using culture medium 50% v/v, centrifuged at 450g x 6min,

supernatant was discarded and resuspended pellet used for cultured in tissue flask T25 (Sigma Aldrich, UK).

5.2.1.2 Passaging the cells.

Passaging the pKer was done, when cell reached approximately 80% of confluence, for further experiments. From the tissue flask medium was removed and 2 ml trypsin (Sigma Aldrich, UK) added to trypsinase cells. Flask was left for a ten minutes in incubator 37ºC, constantly checking under microscope, when cells lifted from the flask surface. When cells not attached to the flask floor, culture medium added to neutralise trypsin 50% v/v. All volume of the flask transferred to the universal tube and centrifuged 2000 x 5min, fluid removed and pellet resuspended in FCM (fibroblast culture medium), centrifuged again, and resuspended before transferring to the culture flask for the further culturing.

5.2.1.3 Counting cells.

For the experiments cells counting perform by using haemocytometer (Hausser Scientific, Horsham, PA, USA). When cells reached confluence, they were trypsinaisd, mixed well in the universal tube and 500µl was taken for cell counting. Haemocytometer was cleaned with 70% ethanol and dried and cover glass placed on the haemocytometer. 20 µl of cell solution added on the edge of one chamber and haemocytometer placed under microscope, focused on grid lines in chamber. Cells were counted in each square in both chambers. The area for each square is $1mm^2$, and cells number was calculated:

$$C = n \times 10^4$$

Where n is the average cell number of cells of the eight squares

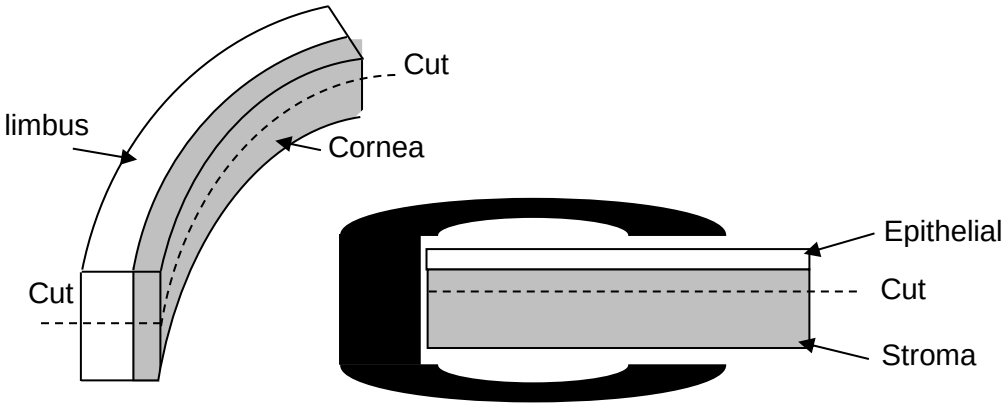


Figure 26 Corneal rim preparation.
Figure shows the level of cutting and separating corneal rim into epithelial part, which used for growing primary epithelial cells. And stromal part, used for growing primary keratocytes.

5.2.2 Corneal epithelial cell line culture

ihCEC (immortalised human corneal epithelial cells, passages 19-26; a kind donation from Araki-Sasaki, Japan[327]) were expanded in EpiLife basal culture medium (Invitrogen, UK) supplemented with 20% v/v FBS, 2.5 µg/ml Plasmocin™ (Autogen Bioclear, UK) 0.02 µg/ml gentamicin and 0.5 ng/ml amphotericin B (combination, Gibco, Invitrogen, UK). Cell cultures were maintained at 37°C under 5% v/v CO2. Culture medium was replaced every 2-3 days until cells were 80% confluent. Cells were passaged at 80% confluence at a 1:3 ratio. Where necessary cells were passaged using TrypLE™ Express (Invitrogen) and cell counts were carried out using Trypan Blue (Sigma-Aldrich).

5.2.3 Human lymphocyte isolation

For human lymphocyte isolation, venous blood from 3 separate donors was used. Human lymphocytes were obtained from the human blood, which collected from the researchers in the department of ophthalmology and visual sciences. Blood was collected with anti-coagulant. Anti-coagulated blood was layered on to 3ml of pre-warmed Histopaque-1077 (Sigma-Aldrich) in 50ml tubes. The sample was then centrifuged at 400 x g for 30 minutes. The interface layer containing lymphocytes was aspirated using a Pasteur pipette and transferred to a separate sterile tube. DPBS (10 ml) was added to the sample and further centrifuged at 250 x g for 10 minutes. The supernatant was discarded and the cell pellet was re- suspended in 5ml DPBS. Cells were then counted using a Trypan Blue (Sigma Aldrich) exclusion method and either cryopreserved or used directly in experiments

5.2.3.1 Counting human lymphocyte.

Human lymphocytes were counted using haemocytometer, as described above.

5.2.4 Primary CEC (corneal epithelial cells) cells isolation and culturing.

5.2.4.1 Primary CEC isolation.

Corneal rims were placed into a petri dish with CEM (Corneal epithelial Medium) contains DMEM (Dubelcco Modified Eagle Medium (Invitrogen, GIBCO, Paisley, UK) + foetal calf serum 5% (Invitrogen, GIBCO, Paisley, UK)+ Cholera toxin Type:Inab a 569B Azide free (Quadratech, Epsom, Surrey, UK) + Insuline (Bovine lyophilised (Invitrogen, GIBCO, Paisley, UK) 4mg/ml) +Epidermal growth factor (R&D Systems, UK)+ gentamicin (Hoechst Marion Roussell, Germany) (50µl X 100mls) +DMSO (Sigma Aldrich, Dorset, UK) +Amphotericin (Sigma Aldrich, Dorset, UK) under a dissecting microscope. The corneal tissue was dissected using blade No.22. After, rim was cut in half, (see *Figure 26*) and residual peripheral cornea removed. Forceps were used to hold the rim, epithelial layer facing up, and the tissue was cut laterally to create a 5mm epithelial/stroma flap. A second pair of forceps was used to grip the flap

and the tissue was pulled apart to the split the layers apart. The back aspect of the tissue was discarded and the epithelial layer was cut into 4-5 explant pieces ready for culture.

5.2.4.2 Primary CEC culturing.

Each tissue section (described 4.2.5.) was placed in the centre of the 3.5cm in diameter plates (with gridded squares) epithelial site up and allowed to air dry for 1-2 min, to allow the explant section chance to stick to plate before carefully adding a small amount of CEM to avoid floating of the section. CEM was added using the pastette in amount to allow the section just to be covered Figure 27.

The culture medium was changed every 2-3 days, using a sterile pastette, and adding 1-2ml of CEM (Cornea Epithelial Medium) medium contest described above.

5.2.4.3 Feeding CEC (primary explants)

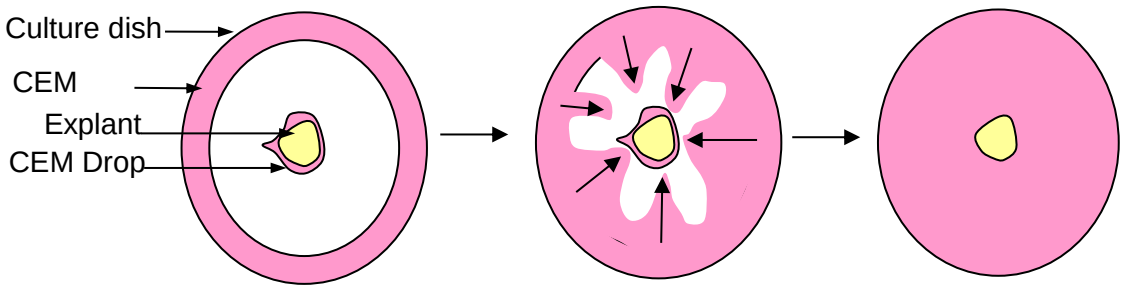


Figure 27 Corneal epithelial cells culture.
Diagram shows corneal epithelial rim attached to the plate with medium around.

Explants were cultured in an incubator and further medium was added after 48 hours incubation. Cell growth was checked and cells were fed every 3 days, Using sterile pastette two third of the old medium were removed and replaced with fresh in the same amount.

5.2.5 Wound healing/Scratch test.

A scratch wound closure assay was performed in vitro five days post seeding, on confluent cultures starved of serum and EGF for 24 hours. A standard single linear scratch with a defined length of 1.6 cm was created in the cell monolayers across each well using a 10 µl pipette tip, giving a 300 µm wound width. Unattached cells were washed away and medium was replaced with SL samples fresh SL, fresh SL with AM and fresh SL without AM (sample preparation see 3.2.2. Spongy layer isolation and preparation). Scratches were photographed immediately (day 0) and then 24 and 48 hours, at four pre-determined positions by phase-contrast imaging at x 100 magnification. Wound healing for each culture was reported as the average linear speed of the wound edge closure over a 48-hours period, using Image software (Wayne Rasband, National Institute of Health).

5.2.6 Apoptosis.

To investigate apoptotic activity (pKer, CEC) cells were treated with different SL samples. After, with forceps covering glass was taken out, washed in cells medium and Corneal keratocytes and corneal epithelial cells were passaged on square shape cover slips (Merck Millipore, Billerica, MA, USA) (as only this type of slips been able to feet into well and take it out for examination under microscope) and placed into 6 well plates (Sigma Algrich, UK), 2000 cells per well. Only first or second passage been used for the experiment. For the first 24 hours, cells were cultured in medium contained 1% serum, however, this was changed to serum free after 24 hours and cells were stimulated with extract of SL. When cells reached 60-70% confluence, culture medium was removed and SL sample added for 12 or 24 hours. Disruption of mitochondrial transmembrane potential is one of the earliest intracellular events that occur following induction of apoptosis. When cells mitochondrial membrane was

destroyed, cell became apoptotic. To detect apoptosis in the cells MitoCapture™ Apoptosis Detection Kit (BioVision, Inc, Milpitas, California, USA) had been used. The MitoCapture™ Apoptosis Detection Kit is a fluorescent-bases method for distinguishing between healthy and apoptotic cells by detecting the changes in the mitochondrial transmembrane potential. The kit utilized MitoCapture™, a cationic dye that fluorescence's differently in healthy vs apoptotic cells. In healthy cells, MitoCapture accumulates and aggregate in mitochondria, giving off a bright red fluorescence. In apoptotic cells, MitoCapture cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and thus it remains in the cytoplasm in its monomer form, fluorescing green. The fluorescent signalling was detected by fluorescence microscopy.

5.2.7 Cell proliferation/ and cytotoxicity

5.2.7.1 WST 1 (Based on the Cleavage of tetrazolium salt by mitochondrial dehydrogenase in viable cells)

For the proliferation assay, cells were passage into 96-well flat bottom plate (Fisher Science, Loughborough, UK). For each well 500 cell were passaged, left to settle and grow for 48 hours, after, culture medium was replaced with SL sample. The cell proliferation reagent WST-1 (Roche, West Sussex, UK) is used for nonradioactive, spectrophotometric quantification of cell proliferation and viability using 96-well format. WST-1 was used to measure cell proliferation response upon stimulation with SL samples. The assay analysed the number of viable cells by the cleavage of tetrazolium salt added to the culture medium. The cells were passaged in flat bottom 96-well plates, volume of cells in culture medium 100 μl/well. As a background control, culture medium without cells was used and all samples were repeated in triplicate.

The 96 well plate was incubated at 37°C, 5% CO_2 for 24 hours. After 24 hours, when cells are settled, SL samples added and incubated for the further 24 hours. 10 μl of premixed WST-1 was added to each well (1:10 final dilution) and incubated for 2 hours. The plate was shaken for 1 min on a shaker, prior to reading on a multiwell plate reader using the 420-480 nm.

5.2.7.2 MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide)

MTT cells growth assay kit (Merck Millipore) was used to duplicate (check) results from WST-1. Experiment done with the same cells types, which were used for WST1 and same SL samples. In a flat bottomed 96-well plate cells (epithelial cell line and corneal keratocites) were seeded and cultured (37°C, 5% CO_2) for 24 hours prior to the assay's commencement, in the incubator. Tested SL samples (sSL – soluble proteins extracted from fresh SL) added 50μl into each well, incubated for 24 hours. Before adding 10 μl of the 12 mM MTT stock solution (component A – prepared 12 mM MTT stock solution by adding 1 ml of sterile PBS to one 5 mg vial of MTT) culture medium was removed and replaced with 100 μl of fresh culture medium and incubated for 2 hours (37°C 5% CO_2). After adding SDS-HCl solution (component B – 10 ml of 0.01 M HCl to the tube containing 1 g of SDS (sodium dodecyl sulphate)), the samples were incubated for 4 hours. Before reading, the sample was mixed using a pipette and the absorbance was read at 570 nm

5.2.7.3 Cytotoxic assay

CytoTox 96® Non-radioactive, colorimetric cytotoxic assay. Quantitatively measures lactate dehydrogenase (LDH), stable cytosolic enzyme that is released upon cell lysis. Tested cells bed passage into 96-well plate with flat bottom (2500 cell/well), after 24 hours SL samples were added to the cells and kept in the incubator for 1 hour. Subsequently all samples were centrifuged for

250xg-10 minutes, and 100 µl of supernatant was removed and transferred into a corresponding well in a new flat bottomed 96 well plate. Reaction mixture – stock solution (freshly prepared) added (100µm) into each well and incubated 30 min before reading the samples at 490-492 nm. Calculation of the results was normalised against the background control (culture medium only), low control (100µl cells in cell culture medium + 100µl reagent), high control (100µl cells with medium + 100µl of 2% Triton X) and LDH control (Positive control).

% of cytotoxicity = average (triplicate reading)-background control-low control/ high control-low control.

5.2.8 Statistical analysis

Results are presented as mean ± SEM. Statistical analyses were performed using the nonparametric Mann-Whitney U test and $p<0.05$ was considered significant.

Statistical analysis was performed with the SPSS package and GraphPad Software, Inc, San Diego, CA). In all WST 1 and CTD assays the mean value of 12 assays +/- standard deviation were calculated. The results were submitted to statistical analysis with the use of SPSS, Version 16.0, statistical software with the use an Oneway Anova or Kruskal-Wallis Tests, accepting $p<0.05$, as significant.

5.3 Results

5.3.1 Cytotoxicity

Cytotoxic assay shows that SL samples have negative effect on cells grow. When CEC and pKer were exposed to Intact SL (iSL) has been shown to exhibit a cytotoxic effect (Figure 28, and Figure 29). Corneal keratocytes and corneal epithelial cells altered their morphology with evidence of cells shrinkage, and vacuolated cells under SL treatment. Cells did not form any colonies, and didn't survive more than 24 hours.

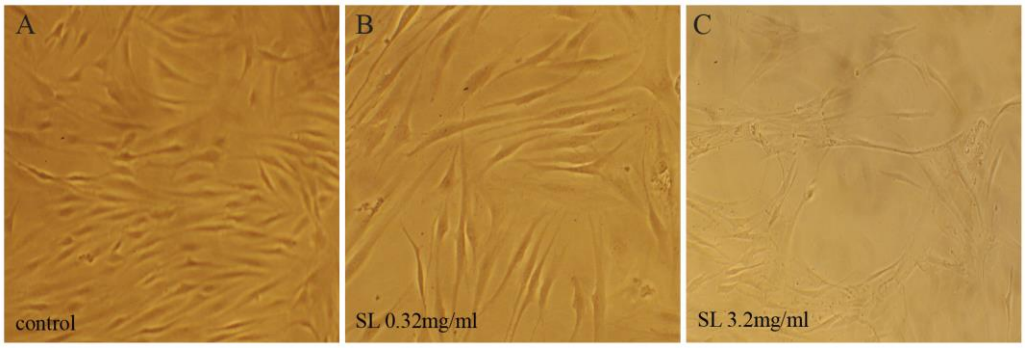


Figure 28 Corneal keratocytes treated with different concentration of SL Figure shows Corneal keratocytes A) control well, cells reached 70-80% of confluence; B) cells treated with SL 0.32mg/ml, confluence much low compare with control plate: C) cells treated with SL 3.2mg/ml very low confluence, not healthy.

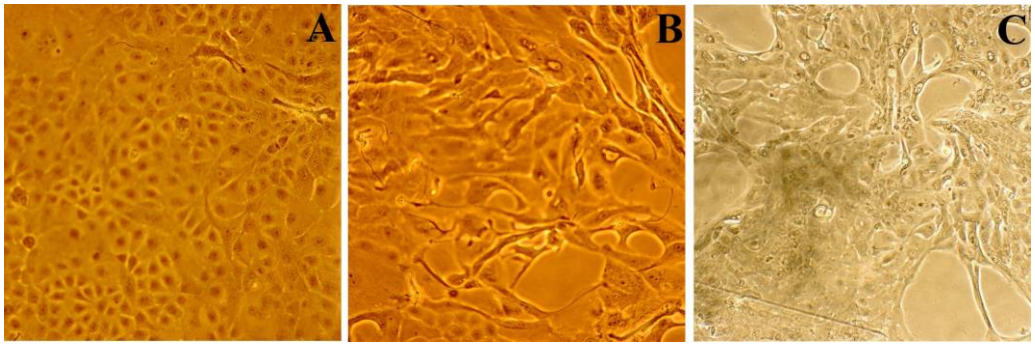


Figure 29 Corneal epithelial cells treated with different concentration of SL. Figure shows Corneal epithelial cells A) control well, cells 80% confluent; B) cells treated with SL 0.32mg/ml, loosing confluence, started to form vacuoles; C) treated with SL 3.2mg/ml, dying cells.

Similarly, human lymphocytes (Figure 30, Figure 31 and Figure 32) were used as an example of non-adherent cells. As from biochemical composition of SL had been detected proteins, which are responsible for immunorespose [328]. From cytotoxic assay results (Figure 28 and Figure 29) SL sample had negative effect on cells grow, human lymphocytes attacked (Figure 30) the fresh whole SL sample and died, as the main function of the T-cells to protect organism (human body) from unknown source, which it current situation SL is. However, figure below (Figure 31), shows that when lymphocytes were treated with SL with lowest concentration of factors 0.32mg/ml, extract and solid SL, result the same, cells died, which is protection reaction from lymphocytes on one hand, and cytotoxic effect of SL, on another.

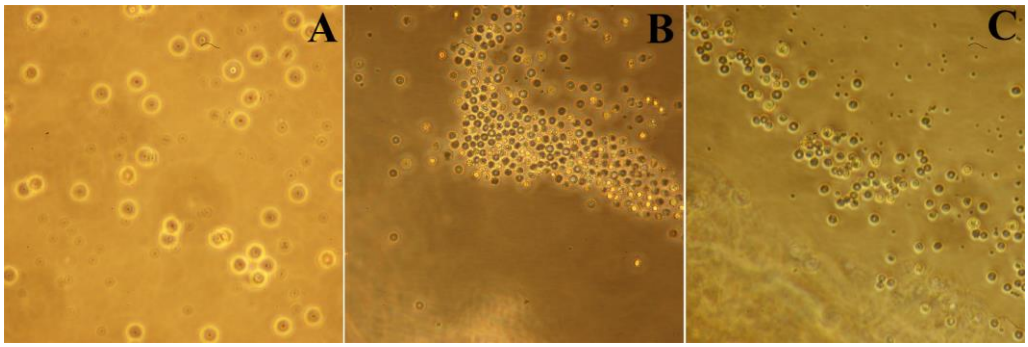


Figure 30 Human lymphocytes treated with different concentration of SL, 24 hours. Figure shows, Human lymphocytes, 24 hours A) control well, cells are healthy: B) cells treated with SL extract 16mg/ml, moved to one area, separated themselves from the SL; C) treated with SL whole 16mg/ml, cells "attacked" the SL, shrinked.

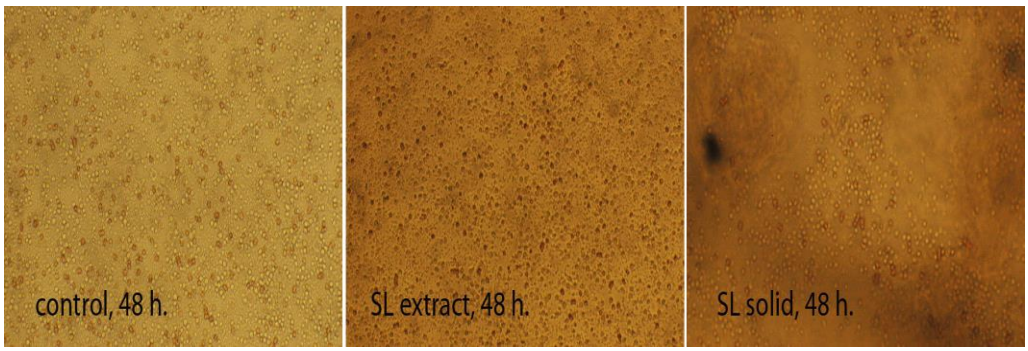


Figure 31 Human lymphocytes treated with different concentration of SL, 48 hours. Figure shows, Human lymphocytes, 48 hours A) control well; B) cells treated with SL 0.32mg/ml, cells not healthy, started to shrink; C) cells treated with SL 0.32mg/ml, cells didn't survived.

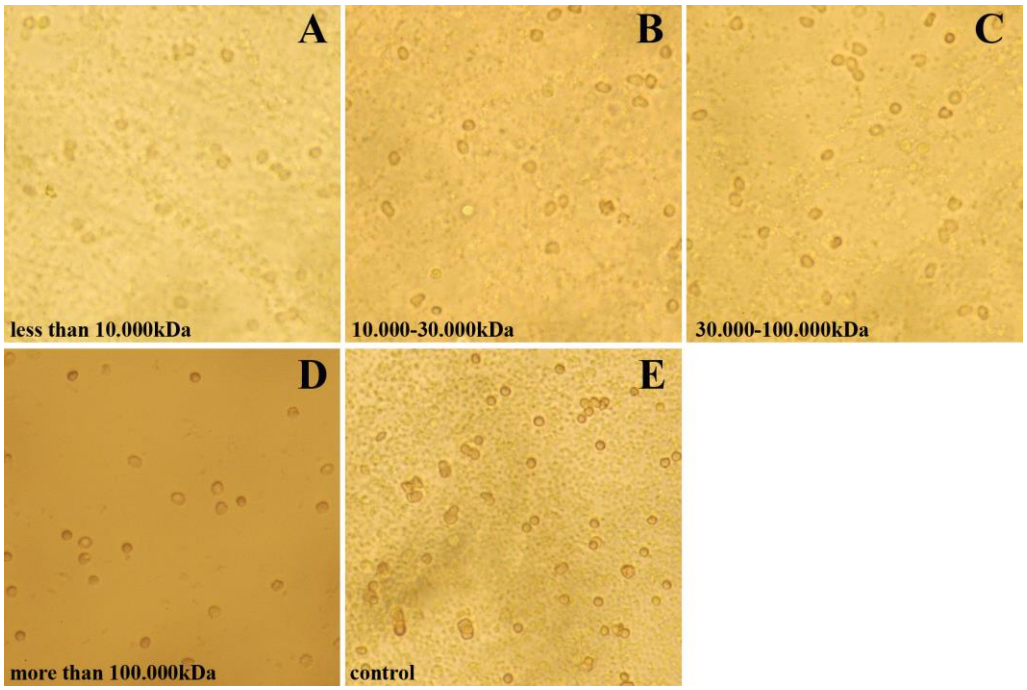


Figure 32 Human lymphocytes treated with different fractions of SL, 24 hours. Figure shows, Human lymphocytes treated with different SL fractions, 24 hours. A) cells treated with fraction contained proteins of molecular weight of less than 10 000kDa; B) cells treated with fraction of SL contained proteins of molecular weight between 10 000kDa and 30 000kDa; C) cells treated with SL fraction contained proteins with molecular weight between 30 000kDa and 100 000kDa; D) cells treated with SL fraction contained proteins with molecular weight more than 100 000kDa; E) control plate.

From the treating the lymphocytes with different SL fractions, separated SL according to the molecular weight of the proteins (Figure 32). Only in fraction contains high molecular weight of proteins lymphocytes did not survived for 24 hours. Which probable means, that cytotoxic effect on lymphocytes had high molecular weight proteins, or lymphocytes did not recognise lower molecular weight proteins as “danger” proteins and did not response.

It may be more clinically effective to remove SL from amnion during AM preparation to increase efficacy. AMT reported to have 70% surgical success rate and 44% recurrence rate [329], This success rate is higher than previously reported [107, 183, 323, 330, 331], however, as AM is a biological tissue with inter and intra donor variation and there are biolovariations in its preparation for AMT. In some techniques, SL not been removed, which means

some AM patches will have significant amount of SL and others will have trace amounts of SL.

Foetus membrane is a barrier between mother and baby is not just anatomical, maternal immune system responding to foetus antigen [332].

Comparatively SL is very similar to WJ. WJ has a high hydration potential and can absorb 98% of its capacity with water. This is a very strong tissue and highly resistant to mechanical damage. It is well known, that WJ has a limited number of cells, but an extensive amount of ECM and high molecular weight components, includingcollagen, glycosaminoglicans, hyaluronic acid and several sulphated proteoglicans [265]. The levels of hyaluronic acid make the tissue highly hydroscopic, and a dense network of collagen makes it highly resistant to extension and compression evoked by foetal movements and uterine constructions [265]. In addition the ECM contains a large amount of FGF and TGF, as observed in our experiments (Chapter 3).

The cytotoxic and proliferative effects of SL on CEC and corneal keratocytes were investigated. SL was cytotoxic to both cell types treated with samples containing all proteins, samples first were diluted into different concentrations, (16mg/ml, 8mg/ml, 4mg/ml) and separated into sSL and iSL(extract) (Figure 8). In comparison and proliferation effect by been treated with samples contained all molecular weight proteins. Fractionated SL samples were tested on the same cells type (Figure 32)., Compare to control plate, cytotoxic activity in plates treated with SL significantly higher, in all proteins sizes. Plates with CEC were treated similarly, and cytotoxic activity on those plates much higher as well compare to control plate (Figure 33).

In addition, CEC and keratocytes were cultured directly on a layer of SL. This approach was highly cytotoxic to the cells within 24 hrs.

5.3.2 Proliferation

Proliferation assay was done with different sets of SL samples. For the first test iSL and sSL samples were used with different concentration of the samples. All tests were perform with CEC and KFBs. Results show the positive effect on both cells type SL samples with concentration of 0.32mg/ml and 3.2mg/ml and negative effect of samples with higher concentration of the proteins 8mg/ml and 16mg/ml

The (Table 30) and the (Table 31) positive effect of samples of all fractions excluding the fraction with highest molecular weight (greater than 100 000kDa for both cells type (CEC and KFB) SL contains multiple range of growth factors, including hEGF (134.28) [271], hHBEGF, hHGH, hSCF(30.0), hTGF α (17.0) [333], hTGF β1 (44.34) [334], hTGF β 2 (47.75) [335], this growth factors are biologically and physiologically important they are responsible for the clinical benefits as they promote proliferation [336]. Majority of those proteins in the fractions with molecular weight range between 30 000 kDa to 47 748 kDa, however, EGF has molecular weight higher than 100 000kDa.

According to the proliferation tests results, SL has increase proliferation of the cells as well as AM[337] [338], which can give a reason to use SL to increase proliferation in clinical site instead of AM or as an additional method.

5.3.3 Wound healing

AM contains important growth factors and biological macromolecules and these have been scientifically documented to be beneficial in reducing pain, suppressing infection and scar formation and providing anti-inflammatory mediators [13, 339-344].

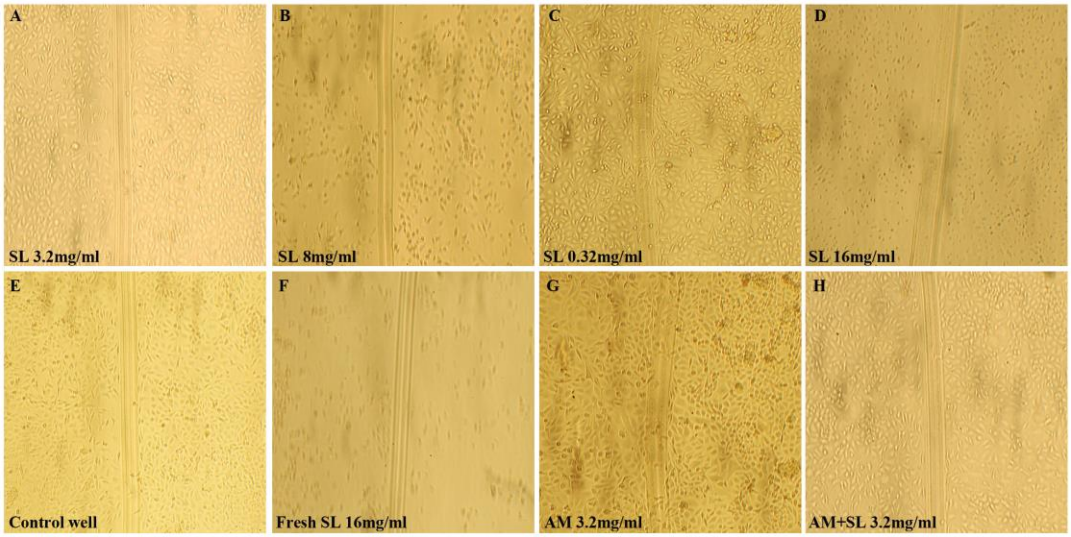


Figure 33 Scratch test CEC.Scratch test done with corneal epithelial cell line treated with different concentration of SL extract, 24 hours; A) cells treated with 3.2mg/ml of SL; B) cells treated with 8mg/ml of SL; C) cells treated with 0.32mg/ml of SL; D) cells treated with 16mg/ml of SL extract; E) control plate; F) cells treated with fresh SL 16mg/ml; G) cells treated with SL extract 3.2mg/ml; H) cells treated with SL extract 3.2mg/ml.

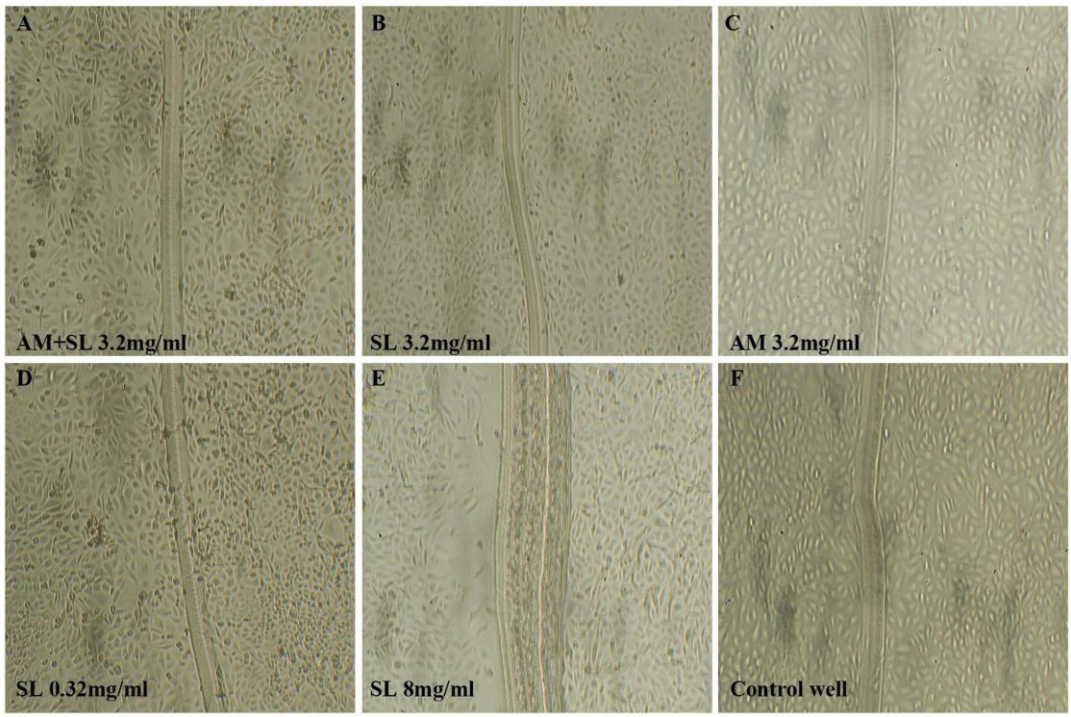


Figure 34 Scratch test KFB. Scratch test done with corneal keratocytes, 24hours; A) cells treated with extract from AM with SL (SL not been separated from AM) concentration of 3.2mg/ml; B) cells treated with SL extract 3.2mg/ml; C) cells treated with AM extract, concentration 3.2mg/ml; D) cells treated with SL extract, concentration 0.32mg/ml; E) cells treated with SL extract 8mg/ml; F) control plate.

For the assays “physiological” concentration of SL was calculated, as in the human body (fetal sac) SL was in it is natural concentration of factors. This calculation was done to make tests on cells as “natural” as possible. The physiological concentration of SL was calculated in proportion of wet weight of SL and dry weight of SL, is 16.35mg/ml (dry weight from one amnion (0.305+0.289+0.273) = 0.289g - average from three single amnions), and volume (21+15+17=17.667ml (average from three single amnions), 289/17.667=16.35 – physiological concentration) therefore 16 mg/ml was used in cellular health assays. From experiments, was found, that SL samples with concentration of 16mg/ml and 8mg/ml have cytotoxic effect, as described above, it was no evidence of cells live, they didn’t forms a colonies, number of cells reduced, and morphology is changed. On Figure 34 A and G cells behaviour is completely different compare to Figure 34 F and D. On the Figure 40 A and G cells are proliferate well, F and D cells are dying. All experiment was performed in three

plicate in time scale 24 hours. AM reported to be clinically beneficial for wound healing [113, 329, 345], as growth factors such as EGF, TGF α, TGF β 1, TGF β 2 play critical role in normal wound healing. About SL can be similar conclusion. In chronic wounds those growth factors have been shown to inhibit mitogenic activity of fibroblasts [336, 344, 346, 347]. As been shown from the project, level of growth factors in SL greater than in AM, which could have been more beneficial in wound healing treatment.

5.3.4 Apoptosis

Apoptosis is a program of cells death, which is related to all cells type. During process of apoptosis, cells shrink and are rapidly eaten by their neighbours, without any debris left.

In contrast, necrosis is uncontrolled cells dead. This process is unpredictable and cells around are not ready, so during some factors, such as injury or inflammation, cells swell and burst, spilling their contents over their neighbours and eliciting inflammatory response [348, 349].

Apoptosis of KFBs was measured by adding a fluorescent marker (described above), and examined through undo immunofluorescent microscope. As a positive control, dexamethasone and mytomicin C was used [79,317, 350-357]

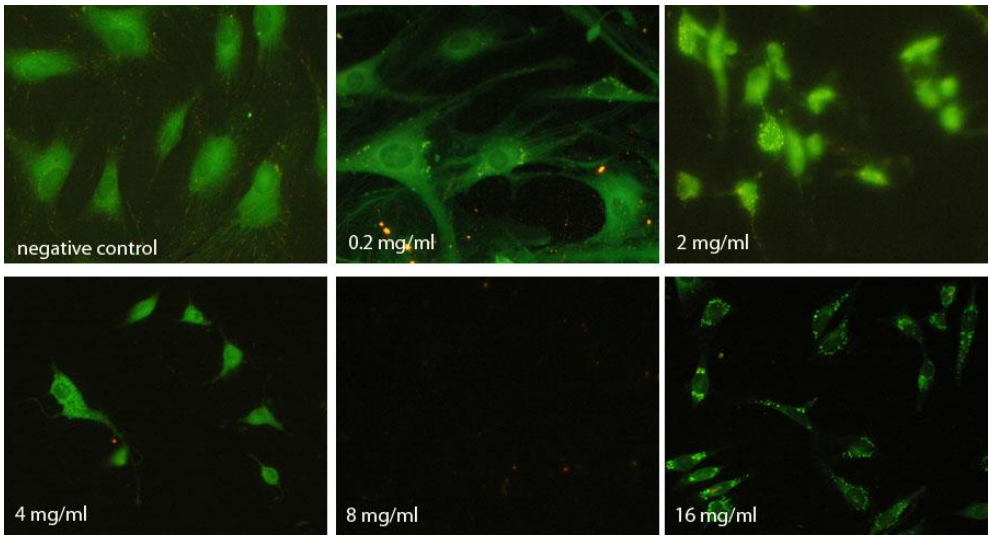


Figure 35 Apoptosis activity of KFB treated with different concentration of SL. Figure shows apoptotic activity of corneal keratocytes treated with extract from SL in different concentration, Negative control shows red staining, which can be in healthy, non-apoptotic cells. All other photographs show apoptotic cells with green staining in mitochondria.

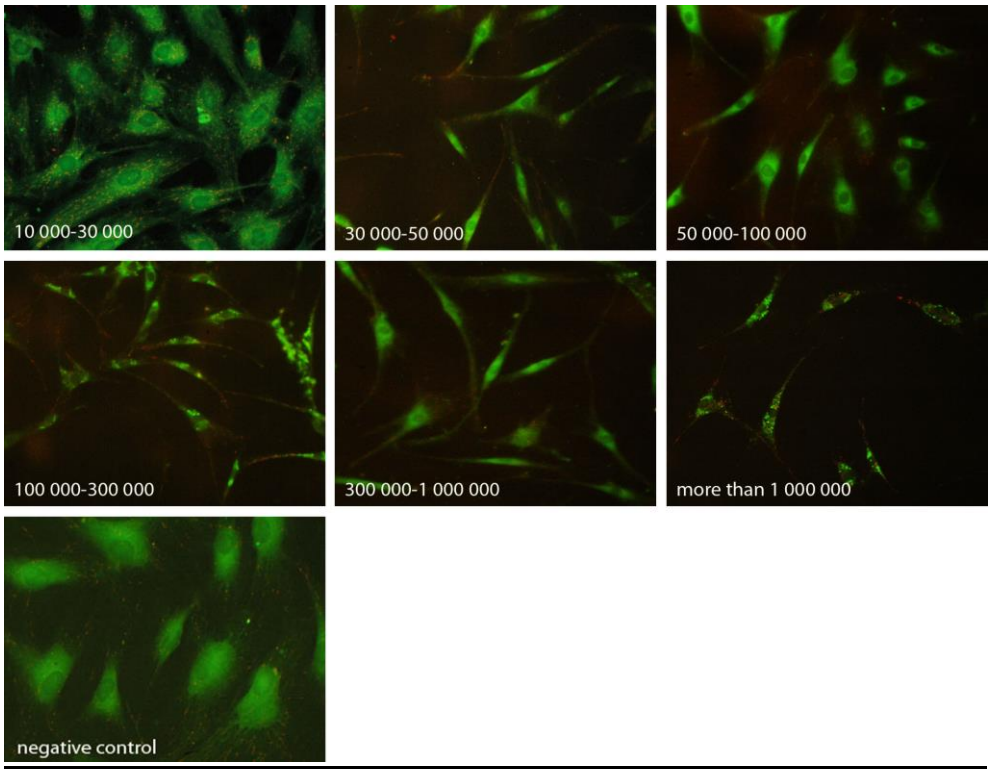


Figure 36 Apoptosis activity KFB treated with SL fractions. Apoptotic assay showing effect of different SL fractions on corneal keratocytes. Red staining in mitochondria seen in negative control and in fraction with 10 000-30 000 kDa only, treatment with all other fractions gave green staining in mitochondria, which detect apoptotic effect.

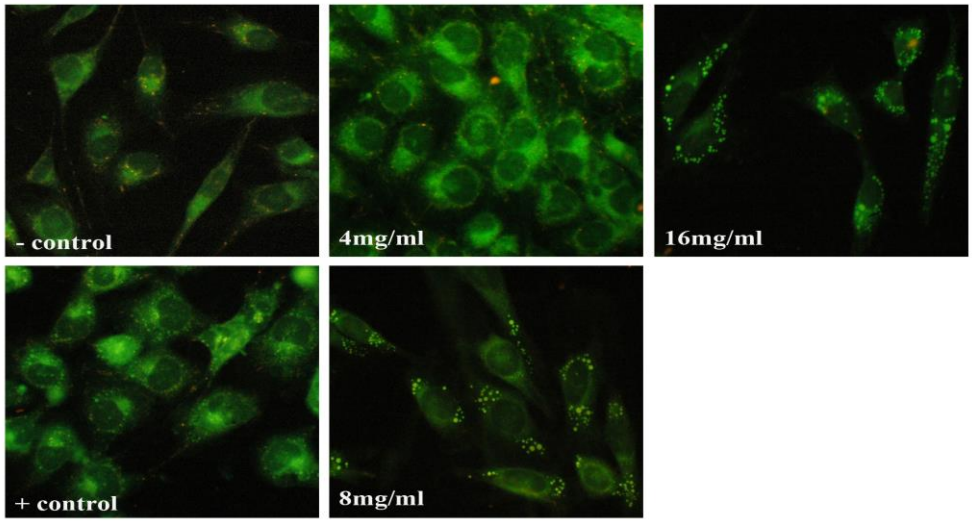


Figure 37 Apoptotic activity CEC treated with different concentration of SL. CEC occur apoptosis under treatment with different concentration of SL extract. Compare – and + controls, in + cells

shape is more round than in – control and clearly green staining in mitochondria, clear red in – control. Under 4 mg/ml SL treatment cells became roundish, but staining is not clear green. Under treatment with 8 mg/ml and 16 mg/ml clear green staining, this is staining of apoptotic mitochondria.

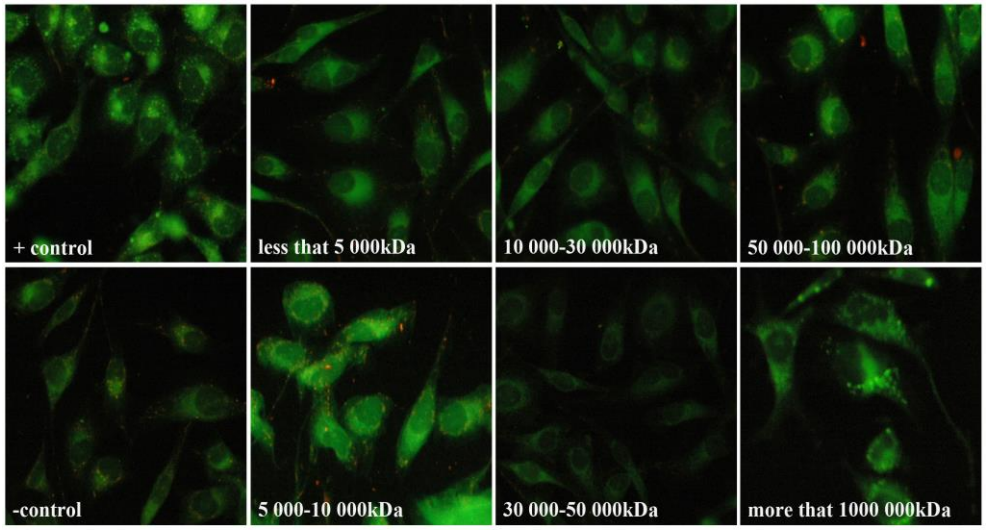


Figure 38 Apoptotic activity of CEC treated with SL fractions. CEC occur apoptosis under treatment with different fractions of SL. Control plates described under Figure 25. Cells treated with fractions of less than 5 000 kDa, 5 000-10 000 kDa and 10 000kDa-30 000kDa didn't changed morphology and mitochondria occur red. Mitochondria of the cells under treatment of 30 000 -50 000 kDa still red, however, cells shape slightly changed to round. Fractions with higher molecular weight of proteins, such as 50 000-100 000 kDa and more than 1 000 000 kDa has clear green staining in mitochondria and cells changed morphology.

The both cells type occur apoptosis under treatment with extract from SL (iSL). The corneal keratocytes occur apoptosis (Figure 35, Figure 36), under treatment with all SL concentrations and corneal epithelial cells in Figure 37 and Figure 38. However, SL fractions with low molecular weight proteins (less than 30 000kDa) do not cause apoptosis, compare fractions with higher molecular weight proteins (30 000kDa and more).

Fractions with molecular weight proteins, which less than 30 000kDa contains proteins such as: IL-8 (8.9), RANTES (7.8), MIP 1b (7.7), BDNF (27.8), CNTF (22.8), MIF (13.8), IL1a (30.7).

Fractions with high molecular weight proteins, which more than 30 000kDa is: IL1a (30.7), TGFb1 (44.3), TGFb2 (47.7), PEDF (50), SCF (55.9), TSP1 (129), Fibrinogen (340). Intact SL (iSL) exhibits a cytotoxic effect. The apoptosis assay, showed that exposure of corneal keratocytes treated with the fractions of SL

which containing high molecular weight proteins do cause the cells death by necrosis rather than apoptosis. However, apoptosis occurs in those corneal keratocytes, which were exposed to fractions with lower molecular weight proteins.

Chapter 6 - Antimicrobial Properties of Spongy Layer

6.1 Introduction

The antimicrobial properties of AM have been previously described in literature [18, 166, 294]. One mechanism described is that AM provides a physical barrier against infection, but the mechanism of how this is achieved is still to be determined

AM has been reported to express anti-microbial peptides [358-360] which are AMPs are expressed at mucosal surfaces by epithelial cells and leukocytes. Those cells are the part of the immune system [165, 361].

AM has natural antimicrobial production which is important to the immune response of the amnion. Primary amniotic epithelial cells produce potent natural antimicrobials, including trappin-2 and SLPI (human secretory leukocyte protease inhibitor – is an 11.7 kDa cationic protein and a member of the innate immunity-associated proteins), which protect pregnancy from infection [166]. The beta-defensin, and beta-3-defensin proved to be present in amniotic epithelium cells and to play a role in the innate immunity of the amniotic cavity [361].

AM has been successfully used as a bandage and as a reservoir for antibiotics. Mencucci *et al* [362] has shown that AM can absorb the antibiotic netilmicin or ofloxacin and in the future AM may be used to deliver antibiotics. These provide a promising method for the treatment of disease caused by corneal endothelial disorders [156, 363]. Because AM acted as a slow release device for up to 7 hours in vitro, depending on the duration of pre-treatment [162] of AM, this could increase the beneficial effects of AMT and the treatment of infectious keratitis.

Considering the reported antimicrobial property of AM, and the discovery that SL acts as a concentrated reservoir of trophic factors, and antibiotics my hypothesis was that SL may also possess a potent antimicrobial function. Therefore, this property was explored further.

6.2 Methods and Materials

The *in vitro* antimicrobial properties of AM and SL were assessed by calculating the minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) Figure 40, against a panel of gram positive and gram negative microbes commonly detected in ocular pathologies; *Staphylococcus aureus (G +), Staphylococcus epidermidis (G+)*, Diphtheoid (Corynebacterium diphtheriae or Pseudodiphteria, G+) and Moraxella catarrhalis (G-) MIC and MBC values were determined with different concentrations of SL extract. All microorganisms were received from department of Microbiology University of Nottingham, UK. SL samples were prepared according to established technique (double liquid nitrogen grinding with two washes in TBSTx).

6.2.1. Preparation of bacterial lawn to be treated with fresh AM with/without SL.

Lawns were passaged on Columbia Agar +5% Horse Blood Petri dish (Life Science BIO-RAD Laboratories Ltd, Bio-Rad House, Hemel Hempstead, Hertfordshire, UK), using four different microorganisms: Moraxella, Staphillococcus Aureus (Staph. A), Streptococcus epidermidis (S. epid.) and Diphteroid were used in the following assays. Microorganisms were received from Department of Microbiology University of Nottingham. Under sterile condition (laminar hood), using sterile microbiological loops, loopfulls of four different bacterial microorganisms were passage into agar plate and let grown for 24 hours in the CO2 incubator 37°C, after three different SL sample (SL only, AM with SL removed, SL facing down, AM with SL removed with SL facing up.) were placed on plates with microorganisms and effect measured in 24 hours.

6.2.2 Minimum Inhibitory Concentration (MIC)

MIC is the minimum concentration of a test substance to inhibit the visible growth of the bacterium in a defined solution MIC is a well established test for biostatic activity of liquid antimicrobials [364]. Medium (described above) did not contained

any antibiotics and serum. The concentration of a defined microorganisms was identified an optical density at 0.1, which is equivalent to 1E8 cfu/ml.

In a 96-well plate, 50 μl of broth medium (Brain-Heart infusion broth (Oxoid, Ref BO 0366D)) was added to each well in triplicate per experiment.

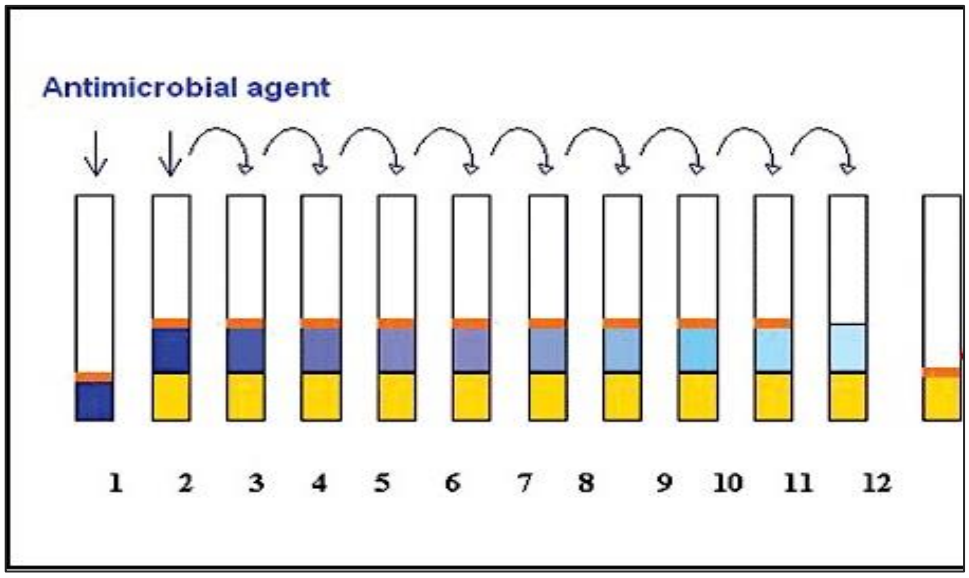


Figure 39 Scheme of dilution method. [365]

The serial dilution of iSL sample was done with started concentration of iSL 4mg/ml Figure 39. In the first column 50μl of undiluted SL extract was added. With multichannel pipette the contents of each well were thoroughly mixed, and then 50 μl been taken to the next well column. Those steps were repeated till reached column eleven (out of twelve). Pipette tips were discarded after each well mixed. Last twelve's column was left with medium only. Suspension of each tested microorganisms with starting concentration of 1E8 ($10mm^3$), was added into each well using a multichannel pipette. After, plate was sealed and kept into a sealed box with secured fixed into a shaking incubator, for 12 hours (overnight) at 37°C at 200 rpm. Examined plate by comparing wells opacity, well in column twelve (last) was visibly cloudy. In each row cloudy and clear wells were recorded. And MIC is calculated as equivalent to the last clear well in a row.

When the well X was clear and well Y cloudy, the concentration of the SL sample in well X is the MIC. As started concentration of the SL sample was known, (in

terms of protein concentration), in the first well SL concentration was 16 mg/ml/4 = 4mg/ml, or 1/4.

6.2.3 Minimum Bacterial Concentration (MBC)

MBC test perform to measure lowest concentration of SL, which will kill bacteria. Test undertaken after MIC, as MIC test did show minimal SL concentration, which will stop bacteria to grow, but not necessary to kill bacteria. To establish the MBC, 10 μl from the first cloudy well (MIC) and from the adjacent three clear wells been taken and placed on an appropriate agar plate in a small grid of four on one quarter of the plate (four determination was done on one plate). In quadrants, where were no growth in the spots from the clear wells then MBC was the same as MIC. In quadrants, where were only first clear well yields growth then MBC is one log lower than MIC. All tests were performed in triplicate.

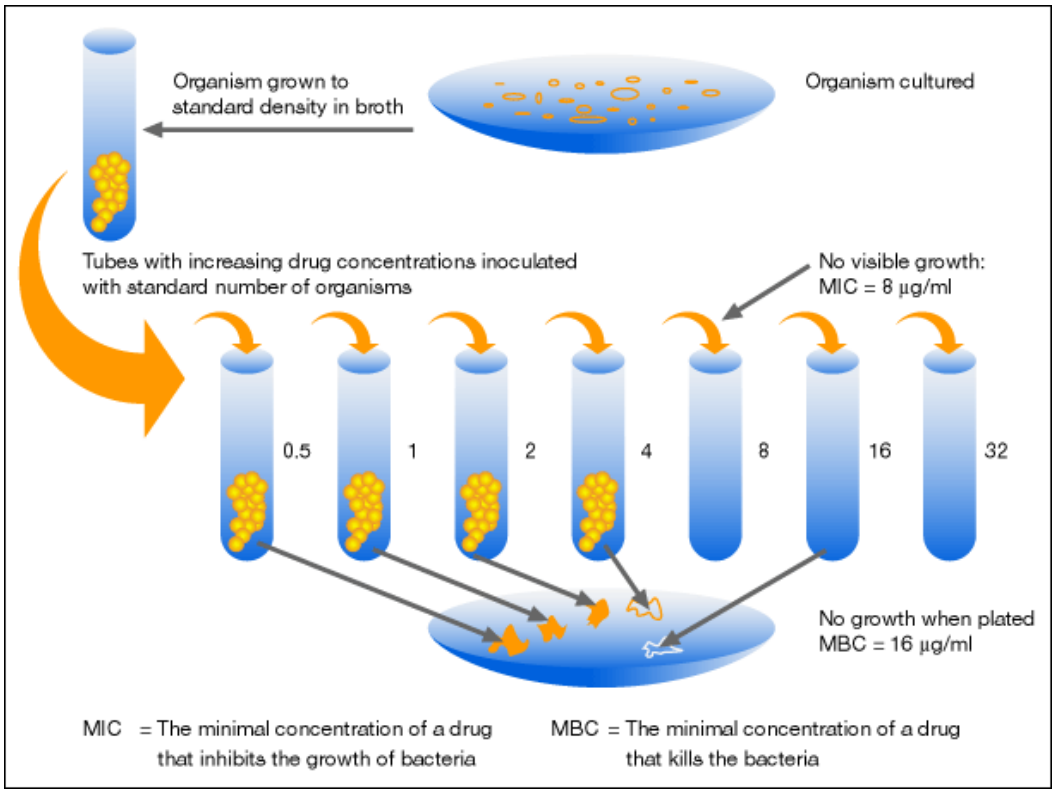


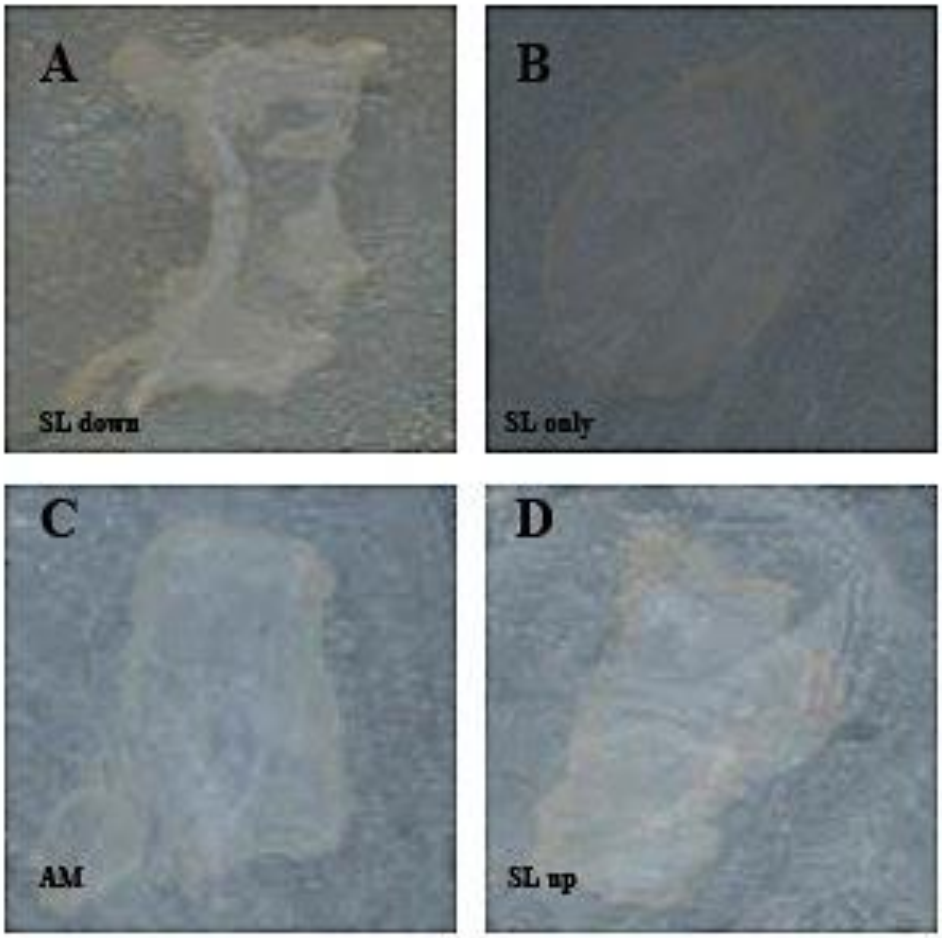
Figure 40 Principe of antimicrobial testing, MIC to MBC.

6.3 Results

In this study SL was found to be bacteriostatic and therefore have antimicrobial properties as an independent layer.

6.3.1 Fresh AM with/without SL on bacterial lawn

All AM containing samples (AM alone, or AM + SL) demonstrated no detectable antimicrobial activity. However, SL only samples when SL removed from AM, such as whole SL, iSL and sSL, prevented bacterial growth. The same experiment was repeated with bigger samples with Moraxella only lawns Figure 42, but used four samples such as A) AM with SL removed placed SL site down; B) SL only; C) AM after SL been removed; D) AM with SL removed placed SL site up Figure 42. In plates A, C and D AM was present, only B plate had no AM at all. In 24 hours of incubation, C and D samples show no difference in bacterial growth. A and B samples show reduce in growth, which can be bacteriostatic or bacteriolytic effect.


A
SL down
B
SL only
C
AM
D
SL up

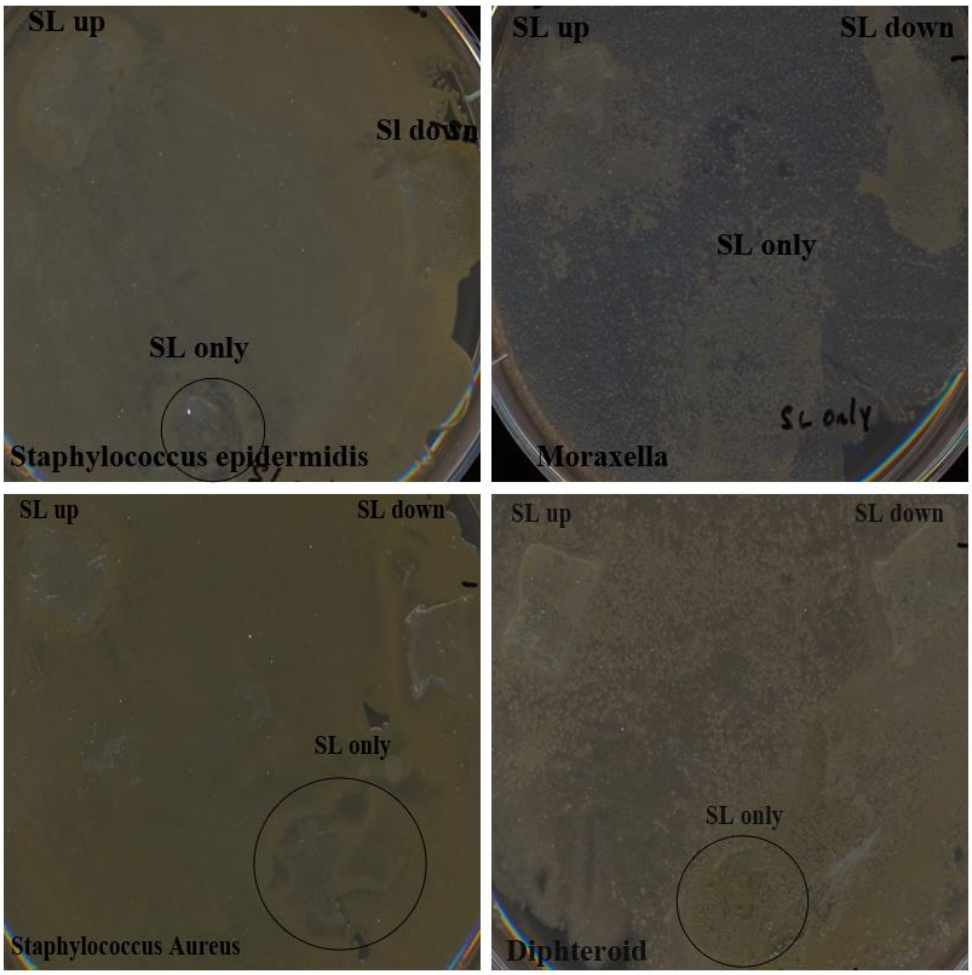


Figure 41 Fresh AM with/without SL on bacterial lawn.
Agar plate lawn with four different microorganisms: Staphylococcus Aureus; Moraxella; Staphylococcus epidermidis; Diphteroid. On each lawn three SL samples were placed: 1) AM with SL attached, SL side up; 2) AM with SL attached with SL side down; 3) SL alone. 1) On the plate with Staphylococcus epidermidis SL only sample shows antimicrobial activity (clear under SL). Samples with AM+SL up or down didn't show any difference. 2) Moraxella plate shows no difference to be treated with AM+SL up or down sides, however, with SL only in the middle of the sample clear spot, which shows antimicrobial activity. 3) Staphylococcus Aureus again AM+SL up or down samples didn't show any changes in bacterial activity, SL only sample did show clearens. 4) Diphteroid plate treated with all three samples AM+SL up and down and SL only didn't show any antimicrobial activity.

	Staph A	Moraxella	S.epid.	Diphteroid
SL side up	-	-	-	-
SL side down	-	-		-
SL only	-/+	-/+	+	-

Table 7 Results related to Figure 1.This table explained described results. (-) shows no reaction on samples, (+) shows positive reaction on samples placed, bacteria stopped or slowed growth.

The same experiment was repeated with bigger samples with Moraxella only lawns Figure 42, but used four samples such as A) AM with SL removed placed SL site down; B) SL only; C) AM after SL been removed; D) AM with SL removed placed SL site up Figure 42. In plates A, C and D AM was present, only B plate had no AM at all. In 24 hours of incubation, C and D samples show no difference in bacterial growth. A and B samples show reduce in growth, which can be bacteriostatic or bacteriolytic effect.

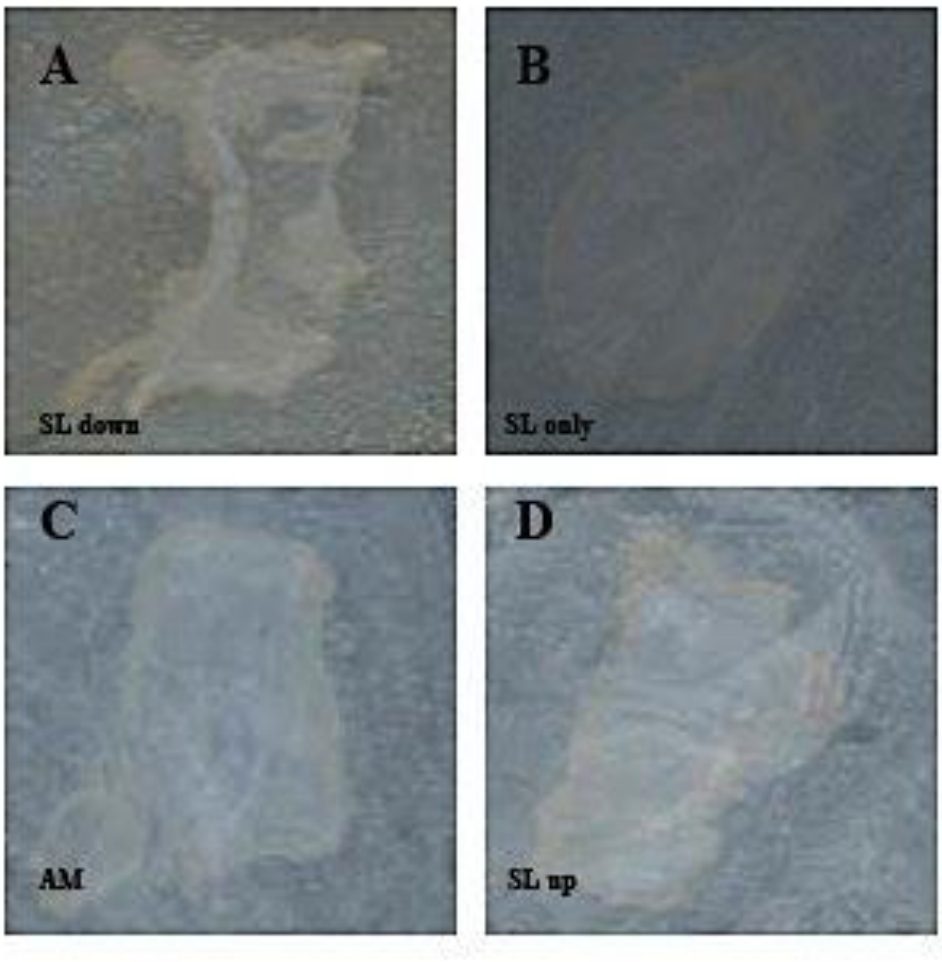


Figure 42 Moraxella lawn with AM and SL. On Moraxella lawn placed AM with SL and without and SL alone: A) SL intact with AM, SL site down, showing clear area in the middle of the sample; B) SL only, shows similar clear area; C) AM without SL, SL was removed; D) SL intact with AM, SL site up.

Lawns with Spaphylococcus Aureus (Staph A), Diphteroid and Staphylococcus epidermidis (S.epid.) did not show any different results from what been described above. However, Moraxella lawn wasn't constant with SL only sample and AM+ SL side down. AM+SL side up and AM only gave constant negative result.

In addition to previously described experiments, SL extract fractions (method described in Chapter 4) were used to treat agar plate with lawns of different microorganisms. Three different sample used A- proteins with molecular mass less than 10 000 kDa; B – proteins with molecular mass between 10 000 kDa and 30 000 kDa; C – proteins with molecular mass more than 100 000 kDa. Plates were kept in incubator overnight at 37°C. The circles on the agar plates Figure 43 with bacterial lawns show the exact place where the drops of SL fractions were placed. In those circles, can be seen area of drops with no difference on bacterial growth. Experiment done in triplicate for all samples and result didn't show antimicrobial activity of chosen samples against four chosen microorganisms. Experiment with different samples was repeated 6 times (n=6).

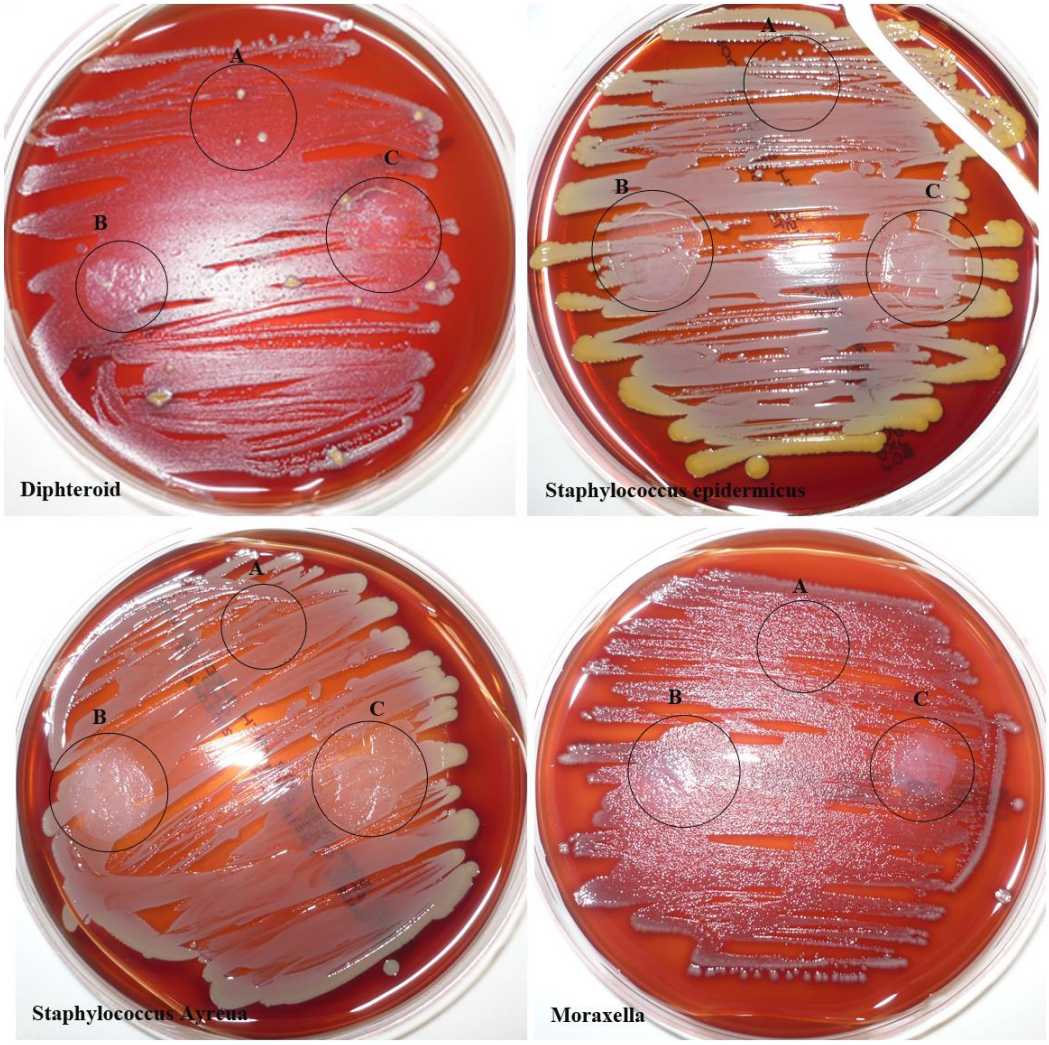


Figure 43 MBC with SL fractions. Four different bacterias treated with SL fractions. On Moraxella, Diphteroid, Staph.Aureus and S.epid. Applied drops of extract of SL in different fractions, A) less that 10 000kDa, B) 10 000kDa – 30 000kDa; C) more than 100 000kDa. All three samples against four different microorganisms didn't show antibacterial activity.

6.3.2 Minimum inhibitory Concentration (MIC)

MIC is a lowest concentration of an antimicrobial sample, which will inhibit the visible growth of the microorganism [366]. Wells containing progressively lower concentration of the sample, and the same amount of the bacteria to each well. For Staphylococcus Aureus gentamicin used as a positive control and MIC of gentamicin against Staphylococcus Aureus 1/32, Sample 1 (Table 8) is a SL sample from one single amnion, Sample 2 (Table 8) is a SL sample from one (another) single amnion and sample 3 (Table 8) is a SL sample pooled from 16

amnions (donors). Antimicrobial activity of SL compare to gentamicin is lower, however, there were donor variations of samples against bacteria [18, 366, 367]

	SL177		SL180		SLPo[16]	
Bacterial Strain	**MIC (%)**	**MBC(%)**	**MIC (%)**	**MBC(%)**	**MIC (%)**	**MBC (%)**
S.aureus	100	100	100	100	12.5	12.5
S. epidermidis	25	25	12.5	12.5	25	25
Diptheroid	50	100	100	100	25	50
Moraxella	12.5	25	25	50	12.5	25

Table 8 MIC of three SL samples compare against gentamycin. MIC of three different SL samples compared against gentamicin. Sample 1 (one single donor), Sample 2 (one single donor), Sample 3 (pool from 16 donors). Highest antimicrobial activity shows samples 1 against S. epid (1/8), Sample 2 against Moraxella (1/8) and Sample 3 against Staph A and S. epid.

6.3.4 TBST/gentamycin/SL

To extract proteins from SL, we used TBST, to exclude effect of left amount of TBST on antimicrobial properties of SL, TBST been tested in comparison with gentamicin and SL samples (SL extract). From clear wells of MIC results, I used samples for MBC. As can be seen on the picture, gentamicin has negative result (control), clear well 1 (SL extract) has grows, but not as much as well 2, TBST has good grow of bugs, with clearly show effect of SL.

6.3.5 MBC

Minimum bactericidal concentration is a lowest concentration of the agent that will prevent the growth of an organism after subculture on to antibiotic free media. After MIC test, from three wells with no growth (clear wells) and last cloudy well 10 μl was placed on agar plate for growing and placed in the incubator for overnight. Each agar plate had four determinations. If there was no growth in the spots from the clear wells, then MBC was equal to MIC (Table9) if only the first clear well yield growth, and then MBC was one log lower than MIC Table 35.

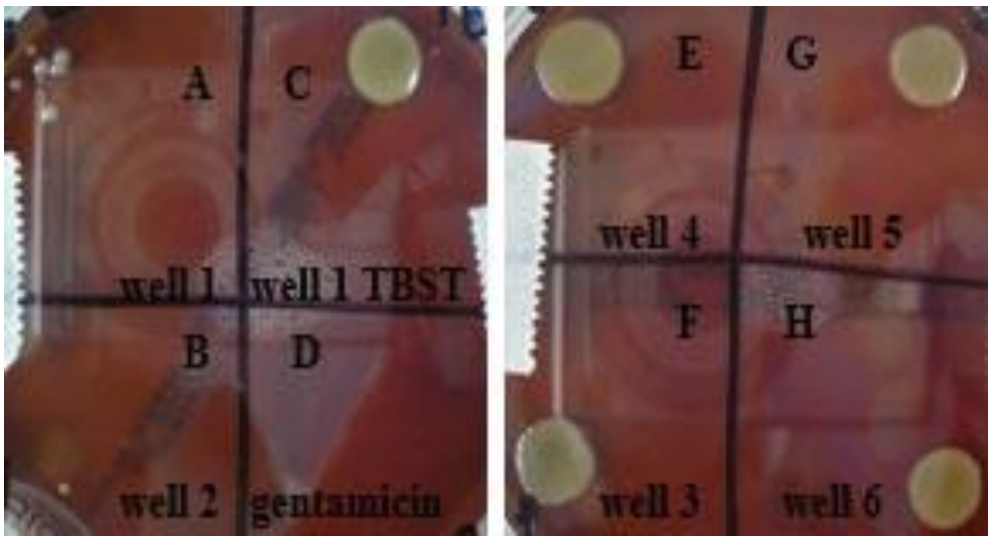


Figure 44 Moraxella treated with SL. A first clear well Moraxella treated with SL; B is second clear plate Moraxella treated with SL; C is passage from well 1 Staph.A treated with TBST only with no SL; D is a positive control clear well Staph A treated with gentamicin; E passage from first clear well Staph A treated with SL; F is second clear well Staph A treated with SL; G passage from third clear well Staph A treated with SL; H passage from fourth clear well Staph A treated with SL.

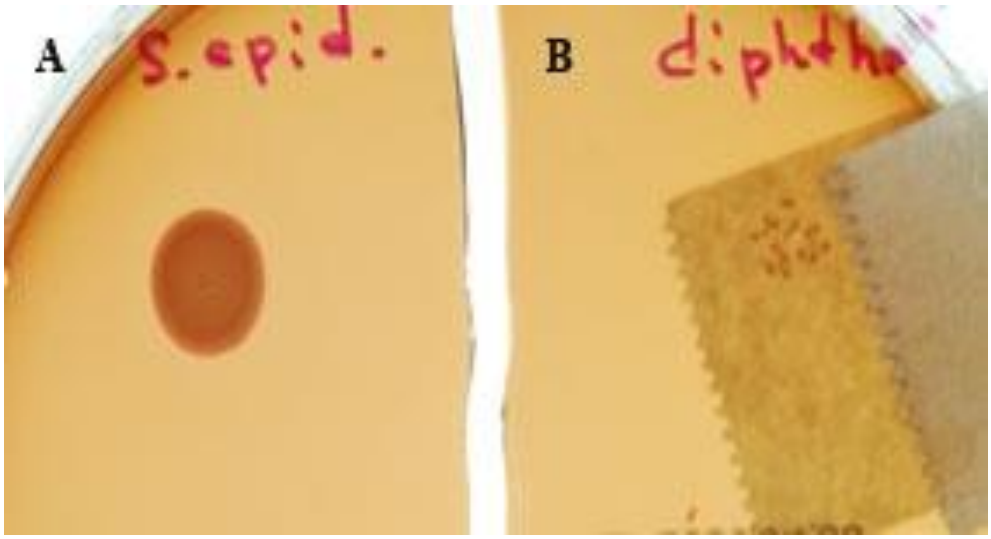


Figure 45 MIC and MBC S.epid after SL treatment A is passaged from clear well S.epid treated with SL; B is a passaged from clear well Diphteroid treated with SL.

	Moraxella	St. A	St. epid.	Dift.
-SL↑	-	++	n/a	n/a
-SL↓	++	+++	n/a	n/a
SL↑	-	-	n/a	n/a
SL↓	+++	+++	n/a	n/a
SL only	+++	+++	n/a	n/a

Table 9 MBC of different SL samples. MBC of different SL samples against Moraxella, Staph A, S. epid. and Diftheroid. Three samples size-effect in cm. sample –SL where SL been removed, arrow up or down shows SL side against amnion. SL only - sample where SL was removed from amnion and amnion wasn't been used.

MIC and MBC Figure 44, Figure 45 shows that SL has antimicrobial effect on microorganisms such as Staph.A, S.epid., Moraxella, Diphteroid. However, as bacterial microorganisms from clear well (MIC) grow on agar plate (MBC), can be concluded, that SL has bacteriostatic effect, not bacteriolytic [368].This founding can have important outcome in treatment with amniotic membrane when SL left attached.

6.4 Discussion

Antimicrobial tests of SL using MIC, MBC, scratch test and bacterial lawn show sample variations, which is important to know, as better results for the samples of pooled SLs, and from technical point of view, the pooled sample is easier to prepare and the amount of the sample is bigger, which gives an opportunity to use one sample for a few applications.

Table 8 and Table 9 show variations of one single SL sample and the pool of 16 samples. In the pooled samples antimicrobial activity was higher, however, on Moraxella and S. epid. The single sample had the same antimicrobial activity. The test Figure 44, shows that SL, has a bacteriostatic effect on bacterials which is similar to antimicrobial effect of AM[18, 165, 166, 294, 361, 362, 367]. Kjaergaard et al reported antimicrobial effect of AM against Staphylococcus aureus [294]. From literature, all reported antimicrobial properties of amnion could have reported the antimicrobial properties of SL, but have not reported the separation of the SL from AM during AM preparation. Figure 42 C, did not show the antimicrobial effect of pure AM, however, Figure 42 A, where AM was used with SL attached, did show the antimicrobial effect.

Clinically therefore, SL can be used as a separate treatment substance, as well as when it is intact with AM, which will have a different treatment outcome (less active antimicrobial, or more active). Treatment of microbial (bacterial) keratitis was successful with AMT. It increased the healing process and decreased corneal haze as well as decreased neovascularisation. Retaining potential antimicrobial properties of AM would be beneficial in the treatment of wounds. When used as a biological bandage AM acts as a physical barrier against microbes, protecting the wound from potential infection and may also exert antimicrobial activity [61].

Chapter 7 – Comparison Between Fresh Amniotic Membrane and the Spongy Layer.

7.1 Introduction

To compare the amount of factors in FRAM (Fresh Amniotic Membrane) without SL, and SL only Search Light assay has been used, described previously. Western Blot and Immunofluorescence had been performed to show the differences and similarities of FRAM and SL. As an addition WJ (Wharton Jelly) was added to the tests to support the theory about the embryological origin of WJ. Table 10, shows the sample number and the factor, which was blocked or stained for.

	Name	**Size**
1	MMP 2	74kDa
2	MMP 3	54kDa
3	MMP9	92kDa
4	IL 8	11kDa
5	ICAM 1	58kDa
6	TGF β1	53kDa
7	CD 29	110kDa
8	CD 34	110kDa
9	CD 44	85kDa
10	CD 45	147kDa

Table 10 Selected proteins for comparison between SL and FRAM. Table represent proteins and their molecular weight, which were selected and tested in SL and FRAM for comparison. Where done Western blot and Immunofluorescence.?

Weston Blot (WB) was the method used to detect proteins in the SL and in AM samples. It used gel electrophoresis to separate nature or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (nature/ non-denaturing conditions). The proteins were then transferred to the membrane where they were probed (detected) using the antibodies specific to the target protein. The proteins of the sample were separated using gel electrophoresis. Separation of the proteins may be by isoelectric point, molecular weight, electric change or a combination of those factors. The nature of the separation depends on the treatment of the sample and the nature of the gel. Polyacrylamide gels and buffers loaded with sodium dodecyl sulphate (SDS) had been used for the work. SDS-PAGE (sodium dodecyl sulphate – polyacrylamide

gel electrophoresis) maintains polypeptide in a denaturated state once they have been treated with strong reducing agents to remove secondary and tertiary structures and thus allows separation of proteins by their molecular weight.

Immunofluorescence staining is the method of staining cells with antibodies. There are two known methods of immunofluorescence staining direct immunofluorescence (DIF) and indirect immunofluorescence (IIF). In my work IIF was used. Stained cells were not directly conjugated to fluorochromes, and second labelled reagent to build primary antibodies was used [369, 370]. IIF has greater sensitivity than DIF. More than one secondary antibody can attach to each primary. As a secondary antibody anti-mouse or anti-rat were used.

7.2 Methods and materials

7.2.1 Western Blot

WB was done with three different samples; 1) AM; 2) SL; 3) WJ. All three samples were prepared (described above), washed three times in PBS, concentrated down using Vivaspin columns (described above) to remove liquid as much as possible. Into each sample loading buffer added (4x NuPAGE LDS sample buffer (Invitrogen, Life Technologies Ltd, Paisley, UK) – 250 µl + 0.5M DTT – 100 µl + ddH_2O – to 1ml). After adding loading buffer samples were incubated at room temperature for 10 min.

To run NuPAGE gel NuPAGE 12 % Bis-Tris 1.0 mm 12 well gels (Invitrogen, UK) were used. Running buffer from stock supplied (20x), for running was diluted to 1x (50ml of stocking solution in 950ml of ddH_2O. When gel was removed from the bag, rinsed in water and comb with paper strip was removed, gel was put in the apparatus and running buffer added till gel is covered. Main time, samples were warmed up to 96ºC for 5 min to allow proteins to denature, before loading gel. Gel running time was 40 min, after gel was transferred to blotting PVDF membrane using transfer buffer (100ml methanol, 50 ml of the NuPAGE transfer buffer (20x Invitrogen, UK) and 750 ml of ddH2O. To transfer gel special layers were created Figure 46: and placed in blotting module.

1	(top)
2	Sponge
3	Sponge
4	Sponge
5	Blotting paper
6	Membrane
7	Gel
8	Blotting paper
9	Sponge
10	(bottom)

Figure 46 Western blot gel layers.

All 10 layers were well soaked in transfer buffer. WB running program 1 hour at 30 V. After blot finished, blotting membrane was placed in a tray with deionised

water to avoid dryness. As a next step membrane was washed in TBST and placed in TBST with 3% BSA to incubate at room temperature for 1.5 hours on a rocking platform, after primary antibody (chosen) was added and left overnight at temperature of 4ºC. After, primary antibody was dried off and membrane washed in TBST 3 times on rocking platform at room temperature and secondary antibody (anti-mouse or anti-rat) was added in TBST +3%BSA in dilution at 1:5000, incubated for 40 min at room temperature on rocking platform, then washed in TBST 3 times for 5 min each wash and developing solution (BCIP/NBT – Colour Development Substrate (5-bromo-4chloro-3-indolyl-phosphate/nitro blue tetrasolium)) added for 10 min and membrane was kept in a dark on the rocking platform. To stopped reaction the membrane was washed under the water 5 min two times.

7.2.3 Immunofluorescence

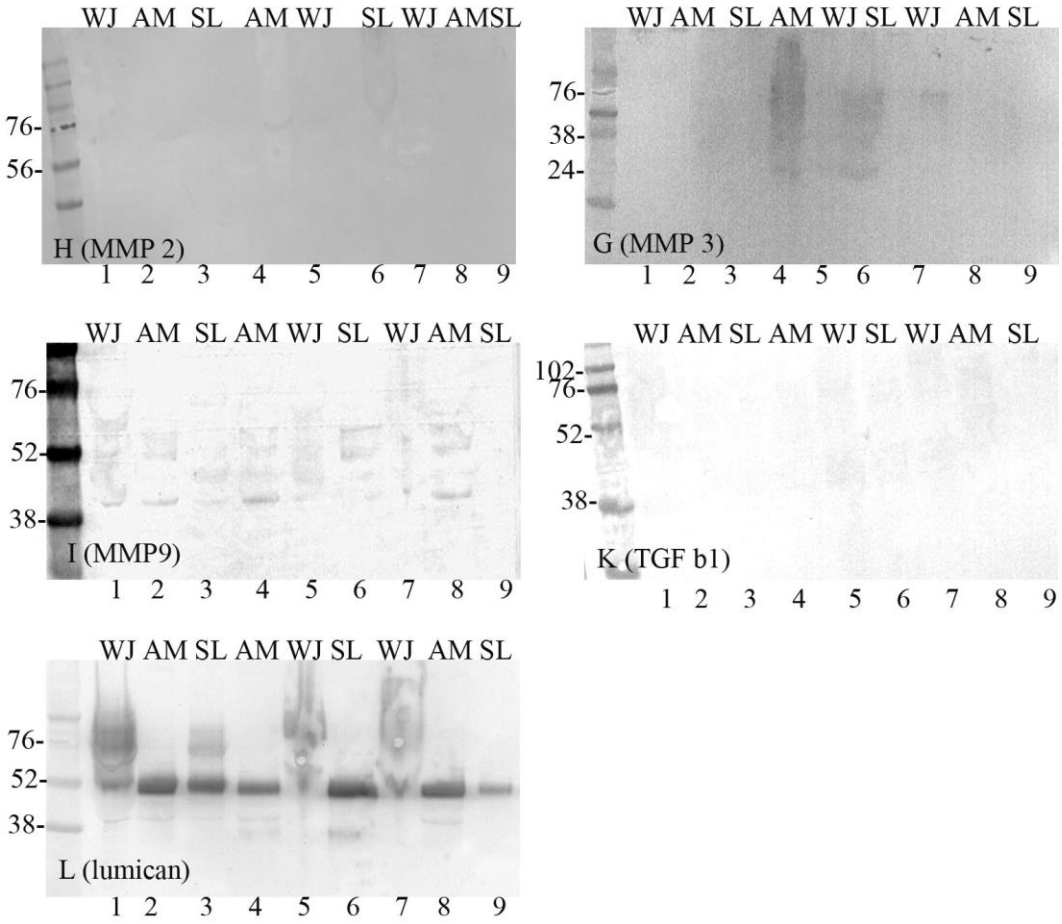
SL sections from different donors were prepared and immunostained [221]. AM, SL and WJ after collection, washing were carefully placed in vertically into pre-moulded aluminium foil cups (1.5-2.0 cm in height) containing cold OCT (Optimal Cutting Temperature) freezing compound (Leica, Germany) and immediately frozen in liquid nitrogen. All samples were stored in the freezer -80°C, prepared samples (10μm sections cut in cryostat (Leica)). Samples were blocked and directly stained with primary antibodies, overnight, at 4°C. Primary antibodies MMP2 (mouse anti-human, ab37150, Abcam, Cambridge, UK) MMP3 (mouse anti-human ab38907, Abcam, Cambridge, UK), MMP9 (mouse anti-human ab51203, Abcam, Cambridge, UK), IL8 (mouse anti-human, ab18672, Abcam, Cambridge, UK) ICAM1 (mouse anti-human ab2213, Abcam, Cambridge, UK), TGFß1 (mouse anti-human, MAB240, R&D Systems®, UK), CD29 (FITC, PN IM0791, Beckman Coulter, High Wycombe, UK), CD34 (FITC, IM1870, Beckman Coulter, UK), CD44 (mouse, MCA89F, Serotec, Kidlington, UK), CD45 (A07782, FITC, Beckman Coulter, UK) were detected using secondary anti-mouse (AF488)

or anti-rabbit (AF488) antibody from Invitrogen,(Paisley, UK). Fluorophore conjugates applied at 1:400 and incubated for 1 hour at room temperature. Slides were counterstained with 4',6-Diamidino-2-phenylindole (DAPI; 1.25 µg/mL; Santa Cruz, Germany). Slides were examined on a fluorescence microscope (Olympus BX51) and imaged using Cell^F software (Olympus, UK). Each experiment was performed in triplicate (Figure 48)

7.3. Results

7.3.2 Western Blot

For Western blot and for Immunofluorescence the same proteins were chosen, to be able to compare. Presenting and amount of MMPs (MMP2, MMP3, MMP9) [293] were tested in Search Light, which had been show in greater amount in SL than in FRAM or fresh AM, the cytokine IL8 not been detected in Search Light assay, however it described in literature, that a factor presented in AM [187, 371] [267]. The cell adhesion molecule ICAM1 [258, 261, 372] is present in all samples in Search Light assay, in SL sample it detected in higher amount compare to FRAM and fresh AM. The growth factor TGFß1 is detected in SL, FRAM and fresh AM in very similar amount, from the Search Light results. TGFß1 expressed in cornea and amnion, which is beneficial for epithelisation after AM transplantation [373] [302]. CD 29, CD 34, CD 44, CD 45, are not been examined in Search Light, however from the literature review, CD 29 and CD 44 are expressed in WJ[374], they are blood delivered and intravascular-derived UC-MSCs (Umbilical Cord – Mesenchymal Stromal Cells) [375, 376]. CD 44 present in WJ [377], it was chosen to show similarities or differences between SL and WJ. CD 34 and CD 45 are haemopoietic markers[374, 376]. CD 34 are expressed in human keratocytes in normal corneas[378], CD 45 in human amnion [374], is presented in AEC, Vosdoganes *et al* shows, that CD45 are play immunological role, which is important from SL site as well as AM [379, 380].


WJ AM SL AM WJ SL WJ AM SL
76-
56-
H (MMP 2)
1 2 3 4 5 6 7 8 9
WJ AM SL AM WJ SL WJ AM SL
76-
38-
24-
G (MMP 3)
1 2 3 4 5 6 7 8 9
WJ AM SL AM WJ SL WJ AM SL
76-
52-
38-
I (MMP9)
1 2 3 4 5 6 7 8 9
WJ AM SL AM WJ SL WJ AM SL
102-
76-
52-
38-
K (TGF b1)
1 2 3 4 5 6 7 8 9
WJ AM SL AM WJ SL WJ AM SL
76-
52-
38-
L (lumican)
1 2 3 4 5 6 7 8 9

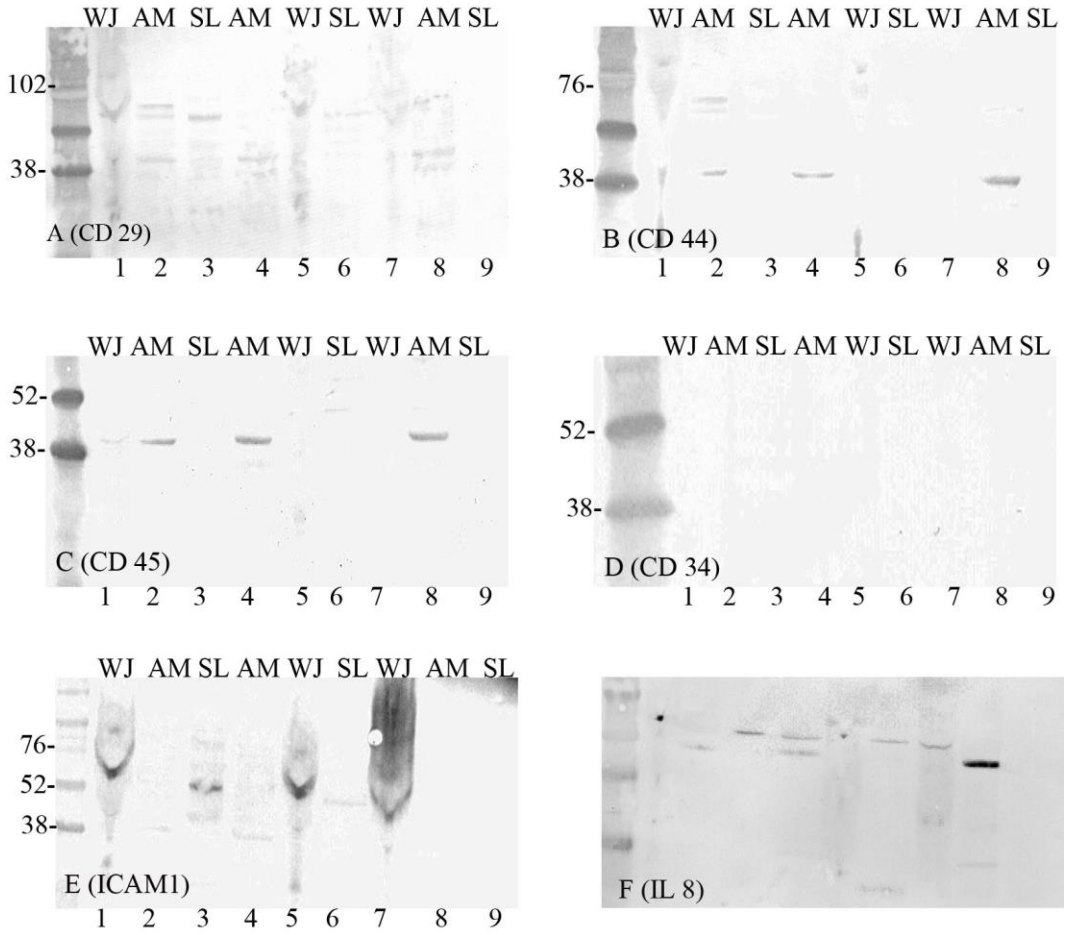
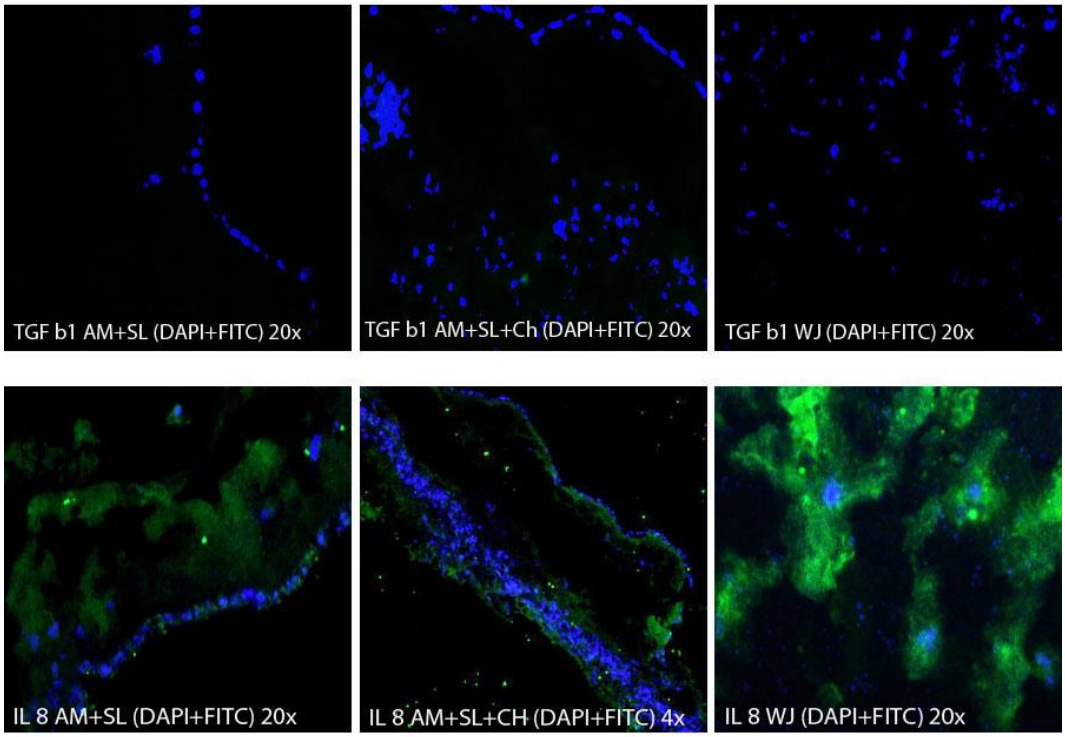


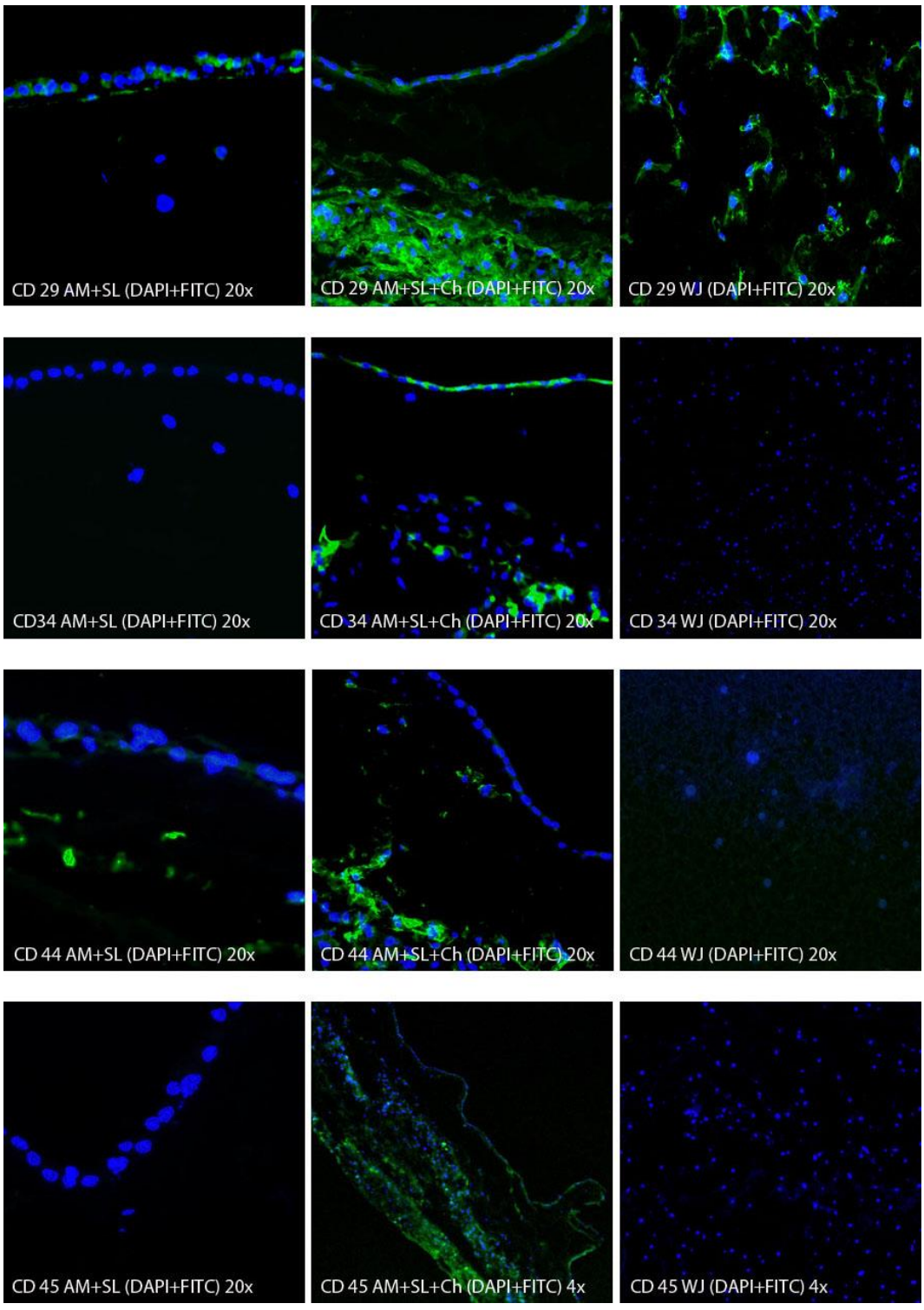
Figure 47 Western blot results. Western blot results three samples (AM, SL, WJ) in triplicate with 11 selected proteins.
First raw or blots shows MMPs staining, for three samples (WJ, AM, SL) in triplicate. MMP2 and MMP3 did not show any staining, MMP9 show weak staining for all three samples in region of molecular weight of proteins between 30kDa and 50kDa.
Second raw of blots and first left blot from the third raw, was stained for CDs, CD 29 and CD 34 did not show any staining, however, CD 44 and CD45 show good staining for AM, around 40kDa, and no staining for WJ and SL.
Second two blots in third raw (ICAM1 and IL8) and first blot in last fourth raw (TGFb1) did not show staining, which cannot be interpreted as a good result of those blots. Last blot for lumican [381] as a positive control show bands in all samples.

The tested proteins Table 10, were blot, Figure 47, and results show, CD 44, CD 45 were in AM only, IL 8 and ICAM 1 in WJ (Wharton Jelly). Proinflammatory cytokines IL 8 and ICAM 1 are expressed in human conjunctival fibroblasts [382], in the tested samples the same cytokines expressed in WJ [383], this can give an idea to use WJ in future as a cell base therapy.

7.3.3 Immunofluorescence

Immunofluorescence done for the same factors as Western blot Table 10 and the same primary antibody had been used, described in 7.2.3 Immunofluorescence was used.




CD 29 AM+SL (DAPI+FITC) 20x
CD 29 AM+SL+Ch (DAPI+FITC) 20x
CD 29 WJ (DAPI+FITC) 20x
CD34 AM+SL (DAPI+FITC) 20x
CD 34 AM+SL+Ch (DAPI+FITC) 20x
CD 34 WJ (DAPI+FITC) 20x
CD 44 AM+SL (DAPI+FITC) 20x
CD 44 AM+SL+Ch (DAPI+FITC) 20x
CD 44 WJ (DAPI+FITC) 20x
CD 45 AM+SL (DAPI+FITC) 20x
CD 45 AM+SL+Ch (DAPI+FITC) 4x
CD 45 WJ (DAPI+FITC) 4x

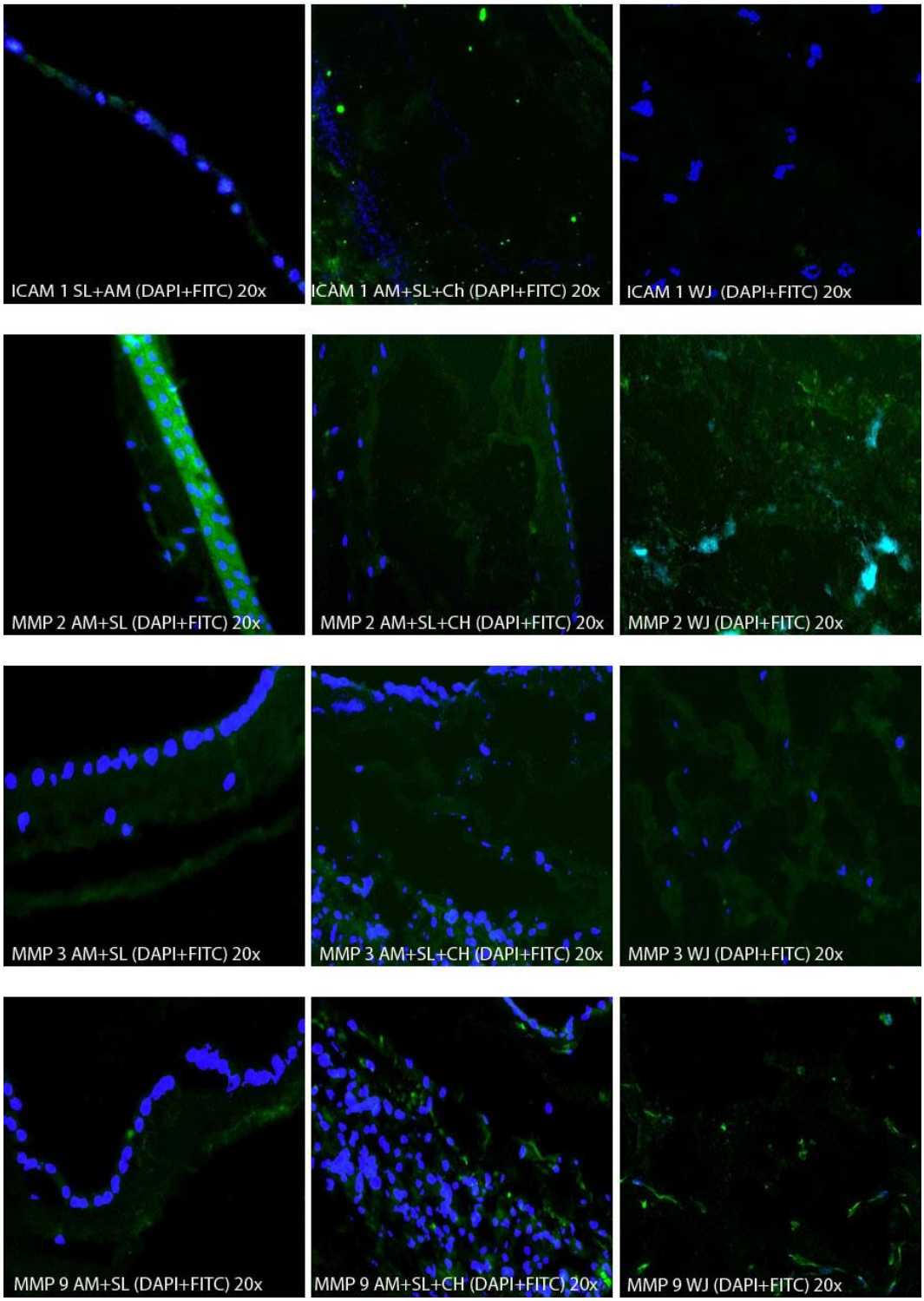


Figure 48 Immunofluorescence staining results. Immunofluorescence staining of 10 selected proteins with AM+SL, AM+SL+Ch and WJ. Each row of images, show staining of specific factors, first image in a row, shows staining of AM+SL; second image in each row show staining of AM+SL+Ch; and last, the third image in a row, shows staining of WJ.

7.4 Discussion

Using the tissue in ocular surgeries may be capable of promoting corneal epithelial cell motility and adhesion. Regulation of the motile or adhesive function may lie with factors secreted by the corneal epithelium that populates the membrane following surgeries. Tested proteins have different functions, this may lie to use AM alone or AM with SL or SL alone in varieties of ocular surgeries depending on the expected outcome.

MMPs were found to play an important role in wound healing and it may be wise to use SL only to increase the speed of wound healing as SL has a greater amount of MMPs compared to AM. In the situation, where the surgeon's goal is to save the eye as an organ for future prosthesis purposes increasing wound healing will form more scaring. Using AM only for wound healing may slow that process however and will decrease scaring which will give a clearer cornea in outcome.

Immunofluorescence images above show the staining of TGFß1, IL8, CD29, CD34, CD44, CD45, ICAM 1, MMP 2, MMP 3, MMP 9 (images from top to bottom) and in three samples such as AM+SL, AM+SL+Ch and WJ (images from right to left). All samples were stained for DAPI and FITC. TGFß1 was stained intensely in SL and in WJ. TGFß1 is stained in SL can be due to secretion into the SL or can be a depot in the SL. TGFß1 stained in amnion [273, 276, 284, 301, 302, 334, 335, 384, 385] and promotes wound healing [386][319, 320, 360, 361]. It is possible that the SL acts as a physiological barrier (described above, Chapter 2), and a physical barrier preventing chorionic TGFß1 from diffusing and infiltrating the AM during gestation. WJ well stained with TGFß1, which could be one of the similarities between SL and WJ. This could be a result of cross - link during embryological development (Chapter 2).

IL8 (second raw, Figure 48) not well stained in SL and WJ, it well presented in AM and Ch, however this interleukin presented in AF [187, 190, 371].

All CDs, (CD 29, CD34, CD44, CD45) (third to sixth raw, Figure 48), when stained in WJ and SL as well as in AM and Ch, which makes difficult to detect original source of the CDs. As CDs are the markers of SC [377, 378, 387] and they are likely to be present in all layers.

ICAM 1 (Figure 48, raw seven) was detected in WJ[388] and AM[258-260, 286, 372, 382, 389].

MMP 2 (Figure 48, raw eight), was not detected in SL and WJ, only in AM [293, 390, 391] and in Chorion.

MMP 3 (Figure 48, raw nine), was detected in all layers[391].

MMP 9 (Figure 48, raw ten), was not detected in SL and WJ, but was detected only in AM and Ch [390, 391] Xu *et al* reported that MMP 9 was localised mainly in amnion epithelial cells, chorion trophoblasts, and placental syncytiotrophoblasts. MMP 2 was localised in amnion mesenchyme, chorion trophoblasts, and blood vessels of placental villi.

Chapter 8 - Conclusion.

The aims of the project were:

1. Identify the embryological origin of the Spongy Layer from the literature review and identify available sources for the research.
2. Investigate biochemical compositions of the Spongy Layer as an independent layer.
3. Establish and optimise the separation of the proteins in the SL and fractionation of those proteins to a standard level.
4. Identify how key proteins contribute to the mediation of wounding and scaring by SL treatment.
5. Investigate the effect of SL on human CEC and KFB lymphocytes.
6. Identify key factors responsible for cell proliferation and cytotoxicity.
7. Investigate the antimicrobial effect of SL.
8. Investigate the differences and the similarities between TRAM and SL.

In my project, I wanted to prove that the SL is a separate layer, which has undeservedly been ignored during AM preparation. This layer has a significant amount of growth factors compare to the AM itself. The SL has been shown to accommodate a complex proteome, which encloses many water-soluble factors.
From the history, the AM has been used for transplantation for more than a century: however the outcome from the AMT use is still not clear.
As mentioned previously, a parallel project was done to the meta-analysis study, which shows the tissue with such a big variation in properties (donor variations and vary laboratory techniques). The donors variation include: age, of donors, number of gestations, medical history, donor's BMI, child's birth weight and sex, blood group, occupations, smoking and treatable pathology, all this variations is difficult to avoid in AMT, however could be minimise in using SL, so AMT cannot be a panacea for multiple diseases with varying aetiologies. The work shows that AMT has sometimes had an unpredictable outcome due to the donor and the

variations in the tissue preparation. Even if variations in the process of preparation are minimized, the donor variations will still be present.

From my study, of biochemical properties, the amnion also can have significant variations according to anatomical (regional) location in one donor [Gicquel, 2009 #97]. Velez et al [Velez, 2008 #279] show the variations in amniotic fluid in ethnic background of the donors [392]. The SL has a vast number of variations in protein content, according to big variations in donors, which gives varied or not expected results in amniotic membrane transplantation. As a result there can be variations in the biochemical properties, especially in the cytokines profile in SL as the cytokine profile of amniotic fluid [393-397] will affect cytokine profile of SL.

In my project, to minimise variations, I combined SLs from 16 AMs (16 different donors). For the further, I would suggest that clinicians probably need to adopt our technique or develop a new of removing SL completely before AMT, as the SL could be the main cause of variations and unpredictable results.

My results show that SL and WJ have very similar properties and form, during the gestation, at the same time, and, both tissues contain a gelatinous substrate, however, quite often the AM, in the process of development continuous to WJ, can cover the umbilical cord as an outer layer. Those tissues are very highly hydrated with water absorbance of 98%. They have a very strong resistance to mechanical damage. It is well known that WJ has a limited number of cells but the amounts of extracellular matrix components are very high. There are a large amount of collagen, glycosaminoglycan, hyaluronic acid and several sulphated proteoglycans also [399]. The amount of hyaluronic acid makes the tissue highly hydrated and the amount of collagen makes it resistant to extension and compression evoked by fetal movements and uterine constructions [170]. Extra cellular matrix contains a large amount of FGF. The main difference is that the UC (umbilical cord) contains MSC (mesenchyme stem cells) because the WJ

develops from the body stalk [383], in comparison with SL, which is from the extraembryonic reticulum [398].

Interesting discovery came from investigating the antimicrobial properties of the SL and would lead to the further investigation to establish time of absorbance of the gentamicin by tissue. The AM reported on antimicrobial [272] (bacteriostatic) properties. Well established research shows that tissue holding antibiotics as a depot for 4-6 hours only, however from my point, that would be the question to answer and compare live tissue and laboratory prepared tissue. It looks possible that tissue should be washed 4-6 hours prior transplanting to disposing the gentamicin from the tissue otherwise the SL or the AM would play a role of depot.

From the book “The scientific basis of Tissue Transplantation”, authors described Spongy Layer, as a fifth and the last layer of the amnion, which is compressed between chorion and developing amniotic sac and is therefore called “stratum intermedium” or “in-between layer”. The SL acts as a visco-elastic pad between the two membranes thus enabling the amnion to move over the chorion, which is attached to the uterine wall. This important function protects the amnion against trauma and rupture during dilation of the cervix in labour.

As previously reported, the SL has eukaryotic cytotoxic effects and this has now been shown to be bacteriostatic. However, the SL is a depot of varying factors with extensive potential effects on cells. It is likely that the SL is responsible for the extensive but variable biochemical composition of transplanted AM and maybe be the key for proteins such as FAST, which are present in the SL in significantly higher concentrations than in FRAM. FAST TNF family kills T cells and activates B cells leading to down regulation of an immune response, which could be an explanation for any cytotoxic effect; and the bacteriostatic effect could have a similar foundation.

The conclusion reached from the wound healing test, was that the AM with the SL attached and the fresh SL has a cytotoxic effect on the human keratofibroblasts and the human epithelial celine. However, fractions containing

large proteins dilate healing process compared with fractions containing middle size and small proteins as compared with the control samples.

As an alternative of removing the SL, after washing, the clean membrane can be prepared directly thereby preserving the SL intact. The functions of the AM with or without the SL can then be assessed experimentally and clinically.

Therefore, in the current situation where the SL is typically ignored during AM preparation for transplantation, and the clinically significant amount of the SL, this may have implications for the clinical efficacy of AM.

Furthermore, it may be important to remove the SL from the amnion during amniotic membrane preparation as it may have a clinically significant effect.

As AMT hasn't got a 100% success rate, I can "guess" that the SL in some situations can be the cause of this problem.

The SL does kill the cells because this is a barrier between mother and baby.

As was observed, the SL has a potential, but variable, depot of beneficial growth factors and their antagonists which effects cell growth differently. Intact SL (iSL) exhibits a cytotoxic effect. From the apoptosis assay corneal keratofibroblasts stimulated with fractions of SL containing high molecular weight proteins do not look like cells which incurs death by apoptosis [400, 401]. More likely it is necrosis. However, those corneal keratofibroblasts, which were stimulated by fractions with lower molecular weight proteins, incur apoptosis

8.1 Conclusion

This study has shown that the SL can be used in clinical practice independently. Also, that the amniotic membrane can be separated from the SL and be used independently, which probably would reduce unsuccessful rate. Or alternatively the SL may be used with the amniotic membrane attached (traditional method).

Summarising my project and discoveries, I would suggest for the future:

1) To investigate the gentamicin staining in the laboratory prepared tissue and its level accordingly to storage time, tissue preparatory technique;

2) To monitor the clinical outcome of the AMT completely free from the Spongy Layer;
3) To investigate the specific protein or group of proteins responsible for the cytotoxicity and apoptosis presented in the Spongy Layer;
4) To compare of WJ and SL properties.

Appendix 1 (Tables)

Factors	***Details***	***Activation/ Mechanisms***	***Factor reported***
Angiogenesis			
Ang2 - angioprotein 2, FGFbasic - Basic Fibroblast Growth Factor, HGF - Hepatocyte Growth Factor (Hepatoprotein), KGF - Keratocyte Growth Factor, TIMP1 - Tissue Inhibitor of Metalloprotease 1, TIMP2 - Tissue Inhibitor of Metalloprotease 2, VEGF - Vascular Endothelial Growth Factor	Inhibit MMPs in a stoichiometric fashion) [236]	Factors lead to rapid destabilisation of local pre-existing vessels and activation of local endothelial cells, a process amplified by further release of growth factors from monocytes, macrophages and fibroblasts [402-405].	ELISAs, [404], RNA [285]
Biomarkers			
FASL - Fas Ligand, Fibrinogen, PEDF - Pigment Epithelium Derived Factor, TRAIL - TNF-related apoptosis-inducing ligand, TSP1 - Thrombospondin 1	FASL death receptor pathway, TNF receptor [406-408]. TSP-1 is a multifunctional matrix protein produced by various cell types exhibiting antiangiogenic activity [408, 409]	FASL induce receptor clustering and formation of a death – including signalling complex [406, 410]. PEDF shown to be most potent endogenous inhibitor of angiogenesis, inducing apoptosis [411].	FASL - Immunohistochemistry [406], PEDF – Western bloting, ELISA [411]
Cell adhesion			
ICAM 1 - Intercellular Adhesion Molecule, ICAM 3 - Intercellular Adhesion Molecule 3, VCAM 1 - Vascular Cells Adhesion protein 1, E-Selectin - Endothelial Selectin	Transmigration of leukocytes from the bloodstream to sites of inflammation. [412, 413]	Oxidative injuries, Induce the activation of the enzyme poly ADP ribose polymerase (PARP), which promote expression of adhesion molecules and the generation of inflammation. [414] [389, 415]	ELISA, PCR [372], immunohistochemistry [413]

Chemokine			
RANTES - Regulated on Activation Normal T cells Expressed and Secreted, MIP1α - Macrophage Inflammatory Proteins (CCL3 – Chemokine (C-C motif) ligand 3), MIP1β - Macrophage Inflammatory Proteins (CCL4 – Chemokine (C-C motif) ligand 4, MIF - Migration Inhibitor Factor	All together, (hRANTES, hMIP1α and hMIP1β has been identified as a natural HIV-suppressive factor secreted by activated CD8+ T cells and other immune cells [416].	Responsible for migration of neutrophils, [417],macrophages inflammatory response.	ELISA [417],
Cytokine			
IFN - Interferon Alpha, IL1α - Interleukin 1 Alpha, IL1β - Interleukin 1 Beta, IL1ra - Interleukin 1 receptor antagonist, IL6 - Interleukin 6, IL8 - Interleukin 8, IL10 - Interleukin 10, TNFα - Tumour Necrosis Factor Alpha, TNFβ - Tumour Necrosis Factor Beta.	The factors reported are anti-inflammatory cytokines, regulate extracellular matrix metabolism and pro-inflammatory[418, 419],	Factors shown to promote further recruitment of polymorphonuclear cells, macrophages, neutrophils, eosinophils. Induce phospholipid metabolizing enzymes and stimulate the ongoing release of prostaglandins, activate ECM, remodelling enzymes involved in a process of human labour. [420]	ELISA [419, 420]
Growth factors			
EGF - Epidermal Growth Factor, HBEGF - Heparin-Binding EGF-like Growth Factor, HGH - Growth Hormone, SCF - Stem Cells Factor, TGF α - Transforming Growth factor Alpha, TGF β 1 - Transforming Growth Factor Beta 1, TGF β 2 - Transforming Growth Factor Beta 2	The grow factors lead to rapid destabilisation of local pre-existing vessels and activation of local endothelial cells. [404]	EGF and EGF-like growth factors increase the secretion of prostaglandin E2. [421], EGF promotes amnion epithelial cells proliferation [422]	ELISA [404], RNA [423], Western blot [421]

Metalloprotease			
MMP1 - Tissue Inhibitor of Metalloprotease 1, MMP2 - Tissue Inhibitor of Metalloprotease 2, MMP3 - Tissue Inhibitor of Metalloprotease 3, MMP7 - Tissue Inhibitor of Metalloprotease 7, MMP8 - Tissue Inhibitor of Metalloprotease 8, MMP9 - Tissue Inhibitor of Metalloprotease 9, MMP10 - Tissue Inhibitor of Metalloprotease 10, MMP13 - Tissue Inhibitor of Metalloprotease 13	The MMPs are important enzymes for the breakdown of extracellular matrix and their activity is regulated by a family of endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) [424].	Secretion of MMPs in AM in response to lipopolysaccharide is mediated by TNF α and ILβ acting in an autocrine/paracrine loop. Molecular mechanism of PROM involve the activation MMPs [291].	ELISA [291], western blot, immunohistochemistry [424, 425]
Neurotrophic factors			
β-NGF–β-Nerve Growth Factor, BDNF - Brain Derived Neurotrophic Factor, CNTF - Cilliary Neurotrophic Factor, GDNF – Glial cell Line-derived Neurotrophic Factor, NT3 - Neurotrophin 3	Been demonstrated, that HAEC (human amniotic epithelial cells) synthesize and release neurotrophic factors (NT-3, BDNF, NGF (nerve growth factor)) [426]	The biological effect of neurotrophic factors are mediated by a common pan-NT low affinity receptor (p75NTR), and one of the three high-affinity, tyrosine kinase-transducing receptors (TrkA, TrkB, and TrkC) [427].	RNA [285], ELISA, immunostaining [427]

Table 2 **Summarising factors involved in biochemical compositions of AM.**
Table represents factors involved in biochemical composition of AM, which been reported in the literature.

Sample Number	***Amnion***	***Details***	***Dry weight***	***Total sample Volume(ml)***	***Concentration Factor***	***Volume (ml)***	***Bradford results (ug/ul)***	***Total protein in sample(µg) in samples sent to Endogen***	***Dilution factor***
1	AM 46	Placenta	0.245	32.5	n/a	0.5	1.897	948.5	65
2	AM 46	Chorion	0.336	33.5	n/a	0.5	0.333	166.5	67
3	AM 46	TRAM	0.048	33	33:3	0.5	0.965	43.86	66
4	AM 46	Fresh	0.233	32.5	n/a	0.5	0.231	115.5	65
5	AM 46	SL	0.167	32.5	n/a	0.5	0.125	62.5	65
6	AM 72	Spongy layer washed	0.237	67	67:6	0.5	0.41	18.36	134
7	AM 72	SL Unwashed	0.082	31	n/a	0.5	0.861	430.5	62
8	AM 72	SL Bloody	0.02	33	33:5.5	0.5	0.324	27	66
9	AM 72	Foetal Blood	0.687	19	n/a	0.5	1.011	505.5	38
10	AM 55	1st wash of AM		350	350:23	0.5	0.541	17.78	700

Table 6 Samples details sent for Search Light.

	hVEGF			hCNTF			hMMP2			hFibronectin		
Details	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)
Placenta	232.1	15087.8	0.2	209.0	13581.8	0.2	47760.5	3104432.5	50.4	13043885.0	847852525.0	13752.1
Chorion	226.0	15142.0	1.4	197.4	13225.8	1.2	39220.9	2627800.3	235.6	5709480.0	382535160.0	34291.2
TRAM	148.0	9770.6	3.4	60.6	4001.6	1.4	3168.5	209120.3	72.2	903915.0	59658390.0	20609.1
Fresh	226.7	14735.5	2.0	165.7	10773.1	1.4	5998.9	389925.3	51.9	3467890.0	225412850.0	30025.0
SL	321.7	20910.5	5.1	252.5	16414.5	4.0	13446.5	874019.3	215.1	30262270.0	1967047550.0	484196.3
Spongy layer washed	210.2	28164.1	11.4	190.1	25468.0	10.4	94414.5	12651543.0	5142.4	27682710.0	3709483140.0	1507772.9
SL Unwashed	183.4	11373.3	0.4	165.4	10253.6	0.4	55455.6	3438247.2	128.8	14970750.0	928186500.0	34775.3
SL Bloody	236.5	15611.0	8.8	174.6	11522.3	6.5	82939.1	5473980.6	3071.8	32523455.0	2146548030.0	1204572.4
Foetal Blood	256.3	9739.8	0.5	338.1	12845.9	0.7	51396.1	1953051.8	101.7	3068230.0	116592740.0	6069.7
1st wash of AM	285.3	199710.0	16.0	494.5	346157.0	27.8	468680.0	328076000.0	26360.0	37955400.0	26568780000.0	2134724.4

Table 7 Original data received from Search Light, and calculated amount of proteins in the samples. (Part 1)

	hPLGF			hHGF				hIGFBP1		hTNFa		
Details	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)
Placenta	13.9	903.5	0.0	1143370.0	74319050.0	1205.5	91955.0	5977075.0	96.94781234	13.5	877.5	0.0
Chorion	<2	0.0	0.0	8741.4	585673.8	52.5	285975.0	19160325.0	1717.567568	8.7	582.9	0.1
TRAM	0.8	52.8	0.0	12406.5	818829.0	282.9	1790.6	118179.6	40.8253534	<2.3	0.0	0.0
Fresh	0.3	52.0	0.0	73980.0	4808700.0	640.5	7396.5	480772.5	64.03896104	33.1	2151.5	0.3
SL	0.2	13.0	0.0	48630.0	3160950.0	778.1	59085.0	3840525.0	945.36	9.9	643.5	0.2
Spongy layer washed	<2	0.0	0.0	39935.0	5351290.0	2175.1	756075.0	101314050.0	41180.55556	42.3	5668.2	2.3
SL Unwashed	5.4	334.8	0.0	12046.1	746858.2	28.0	1533770	95093740.0	3562.764228	6.6	409.2	0.0
SL Bloody	<2	0.0	0.0	10465.2	690703.2	387.6	428810.0	28301460.0	15881.85185	22.0	1452.0	0.8
Foetal Blood	3.8	144.4	0.0	3937.4	149621.2	7.8	65265.0	2480070.0	129.1097923	177.9	6760.2	0.4
1st wash of AM	4.3	3010.0	0.2	53500.0	37450000.0	3009.0	450645.0	315451500.0	25345.61305	49.0	34300.0	2.8

Table 8 Original data received from Search Light, and calculated amount of proteins in the samples. (Part 2)

	hPEDF			hIL1a			hIL2			hIFNg		
Details	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated proteins (diluted)*	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated proteins (diluted)*	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated proteins (diluted)*	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated proteins (diluted)*
Placenta	18130.0	1178450.0	19.1	20.0	1299.4	0.0	5.0	327.6	0.0	5.2	339.3	0.0
Chorion	37031.1	2481083.7	222.4	21.3	1427.1	0.1	5.1	344.4	0.0	6.3	419.4	0.0
TRAM	<29.3	0.0	0.0	14.9	982.1	0.3	0.8	51.5	0.0	0.1	9.2	0.0
Fresh	17571.5	1142147.5	152.1	35.7	2323.1	0.3	4.3	277.6	0.0	5.8	378.3	0.1
SL	31094.6	2021149.0	497.5	24.7	1608.1	0.4	5.1	330.2	0.1	5.6	365.3	0.1
Spongy layer washed	123918.5	16605079.0	6749.4	49.0	6571.4	2.7	8.0	1077.4	0.4	9.0	1200.6	0.5
SL Unwashed	151302.7	9380767.4	351.5	17.9	1106.7	0.0	5.9	362.7	0.0	6.8	418.5	0.0
SL Bloody	37038.5	2444541.0	1371.8	20.6	1356.3	0.8	5.9	388.1	0.2	5.4	355.7	0.2
Foetal Blood	288470.0	10961860.0	570.7	72.3	2747.8	0.1	35.7	1357.7	0.1	12.9	491.3	0.0
1st wash of AM	718105.0	502673500.0	40388.4	29.0	20293.0	1.6	6.5	4557.0	0.4	7.9	5516.0	0.4

Table 9 Original data received from Search Light, and calculated amount of proteins in the samples. (Part 3)

	hLIF			hLeptin			hESelectin			hIL1ra		
Details	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)
Placenta	11.0	711.8	0.0	617.3	40124.5	0.7	650.2	42261.1	0.7	575.9	37434.2	0.6
Chorion	8.5	569.5	0.1	458.4	30714.8	2.8	264.1	17693.4	1.6	813.2	54485.1	4.9
TRAM	1.2	79.2	0.0	115.4	7613.8	2.6	7.9	521.4	0.2	506.9	33454.7	11.6
Fresh	25.2	1637.4	0.2	850.6	55289.7	7.4	215.5	14006.9	1.9	18555.6	1206114.0	160.7
SL	16.3	1061.5	0.3	669.4	43511.0	10.7	177.4	11533.0	2.8	9425.2	612638.0	150.8
Spongy layer washed	23.9	3197.2	1.3	913.4	122394.3	49.7	428.6	57425.7	23.3	10767.7	1442871.8	586.5
SL Unwashed	16.7	1037.9	0.0	641.7	39785.4	1.5	393.1	24371.0	0.9	2306.0	142972.0	5.4
SL Bloody	16.1	1064.6	0.6	540.6	35681.6	20.0	235.1	15517.3	8.7	2209.4	145820.4	81.8
Foetal Blood	167.9	6379.1	0.3	2303.7	87540.2	4.6	8983.4	341368.1	17.8	1897.2	72093.6	3.8
1st wash of AM	34.4	24087.0	1.9	1277.7	894404.0	71.9	905.9	634095.0	50.9	12736.6	8915620.0	716.3

Table 10 Original data received from Search Light, and calculated amount of proteins in the samples. (Part 4)

	hTGFB1			hTGFB2			hBDNF			hICAM1		
Details	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)
Placenta	18172.0	1181180.0	19.2	1835.7	119321.8	1.935392725	187.3	12171.9	0.2	48941.3	3181183.2	51.6
Chorion	1583.5	106094.5	9.5	481.9	32285.96	2.894174174	221.4	14835.1	1.3	5760.4	385944.1	34.6
TRAM	1123.1	74125.9	25.6	176.0	11617.32	4.013223894	249.7	16479.2	5.7	1212.4	80020.7	27.6
Fresh	764.7	49705.5	6.6	133.3	8661.9	1.153766234	245.2	15940.6	2.1	1432.2	93092.4	12.4
SL	1181.8	76818.3	18.9	312.4	20304.7	4.99808	245.2	15940.6	3.9	2886.1	187598.5	46.2
Spongy layer washed	1501.7	201227.8	81.8	517.9	69403.96	28.21023965	245.2	32862.2	13.4	5308.5	711339.0	289.1
SL Unwashed	1484.7	92050.2	3.4	381.1	23625.72	0.885156794	245.2	15204.9	0.6	3832.3	237603.2	8.9
SL Bloody	3914.8	258375.5	145.0	678.5	44782.32	25.13037037	245.2	16185.8	9.1	1712.7	113036.2	63.4
Foetal Blood	1447.2	54995.1	2.9	858.3	32616.16	1.697962413	245.2	9319.1	0.5	14216.5	540227.0	28.1
1st wash of AM	1276.7	893662.0	71.8	438.9	307258	24.68728909	245.2	171668.0	13.8	5737.2	4016040.0	322.7

Table 11 Original data received from Search Light, and calculated amount of proteins in the samples. (Part 5)

	hTIMP1			hTIMP2			hLSelectin			hProlactin		
Details	**Original, from search light hIFNg pg/ml**	**pg/ml of concentrated and undiluted SL**	**normalised; pg/mg against concentrated proteins (diluted)**	**Original, from search light hIFNg pg/ml**	**pg/ml of concentrated and undiluted SL**	**normalised; pg/mg against concentrated proteins (diluted)**	**Original, from search light hIFNg pg/ml**	**pg/ml of concentrated and undiluted SL**	**normalised; pg/mg against concentrated proteins (diluted)**	**Original, from search light hIFNg pg/ml**	**pg/ml of concentrated and undiluted SL**	**normalised; pg/mg against concentrated proteins (diluted)**
Placenta	9714.1	631416.5	10.2	30620.0	1990300.0	32.3	6482.4	421354.1	6.8	255.6	16614.0	0.3
Chorion	7114.6	476678.2	42.7	3993.1	267537.7	24.0	2419.1	162079.7	14.5	250.9	16810.3	1.5
TRAM	2531.4	167073.7	57.7	1554.1	102570.6	35.4	1667.1	110028.6	38.0	3.9	259.4	0.1
Fresh	3340.8	217152.0	28.9	2609.9	169644.2	22.6	1364.1	88663.3	11.8	6.1	393.3	0.1
SL	4164.7	270705.5	66.6	2341.8	152219.6	37.5	1476.0	95937.4	23.6	11.4	741.7	0.2
Spongy layer washed	35025.0	4693350.0	1907.7	6751.9	904754.6	367.8	4398.8	589443.2	239.6	1150.0	154100.0	62.6
SL Unwashed	60070.0	3724340.0	139.5	8212.5	509175.0	19.1	6570.9	407397.7	15.3	6850.0	424700.0	15.9
SL Bloody	8295.8	547522.8	307.3	5030.1	331986.6	186.3	2194.6	144845.6	81.3	38.8	2557.5	1.4
Foetal Blood	26686.8	1014099.5	52.8	8487.8	322536.4	16.8	109347.2	4155193.6	216.3	2185.0	83030.0	4.3
1st wash of AM	132720.0	92904000.0	7464.6	22229.0	15560300.0	1250.2	9444.4	6611087.0	531.2	3230.0	2261000.0	181.7

Table 12 Original data received from Search Light, and calculated amount of proteins in the samples. (Part 6)

	hOPG			hIL1b			hIL3		hTGFa			
Details	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)	Original, from search light hIFNg pg/ml	pg/ml of concentrated and undiluted SL	normalised; pg/mg against concentrated proteins (diluted)
Placenta	21.1	1369.6	0.0	9.3	606.5	0.0	<3.1	0.0	0.0	28.8	1873.3	0.0
Chorion	108.0	7236.0	0.6	11.1	741.0	0.1	<3.1	0.0	0.0	17.1	1147.7	0.1
TRAM	4.3	280.5	0.1	3.3	214.5	0.1	<3.1	0.0	0.0	13.7	902.9	0.3
Fresh	6.7	436.2	0.1	4.1	268.5	0.0	<3.1	0.0	0.0	41.4	2692.3	0.4
SL	3.6	233.4	0.1	3.3	212.6	0.1	<3.1	0.0	0.0	36.6	2381.6	0.6
Spongy layer washed	31.5	4223.7	1.7	8.2	1094.8	0.4	<3.1	0.0	0.0	39.3	5264.9	2.1
SL Unwashed	112.7	6987.4	0.3	10.9	676.4	0.0	<3.1	0.0	0.0	22.8	1411.1	0.1
SL Bloody	23.8	1572.1	0.9	7.7	510.8	0.3	<3.1	0.0	0.0	22.6	1494.2	0.8
Foetal Blood	16.1	612.2	0.0	12.4	470.1	0.0	125.1	4753.8	0.2	54.5	2069.1	0.1
1st wash of AM	17.7	12390.0	1.0	9.7	6790.0	0.5	6.8	4760.0	0.4	118.1	82677.0	6.6

Table 13 Original data received from Search Light, and calculated amount of proteins in the samples. (Part 7)

	hIL4			hIL7			hIL9			hHGH		
Details	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated*	*Original, from search light*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated*	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated proteins (diluted)*	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated*
Placenta	0.5	31.9	0.0	0.2	0!	0	<6.1	0.0	0.0	2438.7	158516.2	2.6
Chorion	0.3	22.1	0.0	0.1	6.7	0.0	13.6	911.2	0.1	274.8	18410.3	1.7
TRAM	1.6	103.6	0.0	<0.2	0.0	0.0	<6.1	0.0	0.0	57.4	3785.1	1.3
Fresh	1.5	94.3	0.0	<0.2	0.0	0.0	6.9	448.5	0.1	71.2	4625.4	0.6
SL	2.3	150.2	0.0	<0.2	0.0	0.0	11.4	741.0	0.2	128.2	8333.0	2.1
Spongy layer washed	1.2	155.4	0.1	0.1	13.4	0.0	<6.1	0.0	0.0	152.6	20451.1	8.3
SL Unwashed	0.2	11.8	0.0	0.1	6.2	0.0	<6.1	0.0	0.0	372.6	23103.7	0.9
SL Bloody	<0.8	0.0	0.0	0.1	6.6	0.0	1.3	85.8	0.0	149.7	9880.2	5.5
Foetal Blood	41.9	1591.8	0.1	12.1	482.6	0.0	992.9	37730.2	2.0	454.6	17276.3	0.9
1st wash of AM	7.5	5250.0	0.4	1.7	1190.0	0.1	6.3	4410.0	0.4	418.8	293153.0	23.6

Table 14 Original data received from Search Light, and calculated amount of proteins in the samples. (Part 8)

	hIGFBP3			hBNGF			hMMP8			hVCAM		
Details	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated proteins (diluted)*	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated proteins (diluted)*	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated proteins (diluted)*	*Original, from search light hIFNg pg/ml*	*pg/ml of concentrated and undiluted SL*	*normalised; pg/mg against concentrated proteins (diluted)*
Placenta	4558.5	296304.5	4.8	6.2	400.4	0.0	8739.9	568091.6	9.2	37295.8	2424228.3	39.3
Chorion	6833.0	457813.0	41.0	1.8	119.3	0.0	1305.3	87457.1	7.8	8255.7	553130.6	49.6
TRAM	1760.9	116221.4	40.1	0.6	40.9	0.0	15.8	1039.5	0.4	1580.8	104332.8	36.0
Fresh	2277.2	148016.1	19.7	0.3	22.1	0.0	93.1	6049.6	0.8	1522.1	98937.8	13.2
SL	2485.6	161566.6	39.8	0.6	37.1	0.0	340.4	22124.1	5.4	5301.2	344576.1	84.8
Spongy layer washed	6417.6	859953.0	349.5	0.6	85.8	0.0	1218.0	163205.3	66.3	10207.4	1367790.3	556.0
SL Unwashed	23565.0	1461029.4	54.7	1.6	101.1	0.0	2948.1	182782.8	6.8	9410.5	583452.9	21.9
SL Bloody	4853.6	320336.3	179.8	1.8	121.4	0.1	3462.4	228515.1	128.2	7363.4	485984.4	272.7
Foetal Blood	5140.7	195345.8	10.2	22.2	842.1	0.0	34173.0	1298574.0	67.6	151399.6	5753184.8	299.5
1st wash of AM	51627.3	36139089.0	2903.7	15.0	10472.0	0.8	1499.5	1049636.0	84.3	52638.1	36846670.0	2960.5

Table 15 Original data received from Search Light, and calculated amount of proteins in the samples. (Part 9)

	hNT3			hIL10		
Details	**Original, from search light hIFNg pg/ml**	**pg/ml of concentrated and undiluted SL**	**normalised; pg/mg against concentrated proteins (diluted)**	**Original, from search light hIFNg pg/ml**	**pg/ml of concentrated and undiluted SL**	**normalised; pg/mg against concentrated proteins (diluted)**
Placenta	3.6	235.3	0.0	0.5	33.2	0.0
Chorion	0.9	60.3	0.0	0.2	11.4	0.0
TRAM	0.5	35.0	0.0	0.1	5.9	0.0
Fresh	0.4	23.4	0.0	0.1	3.9	0.0
SL	0.4	23.4	0.0	0.1	3.9	0.0
Spongy layer washed	0.5	65.7	0.0	0.2	24.1	0.0
SL Unwashed	1.1	67.6	0.0	0.3	21.1	0.0
SL Bloody	1.4	93.7	0.1	0.6	39.6	0.0
Foetal Blood	9.8	372.8	0.0	1.3	50.5	0.0
1st wash of AM	4.9	3458.0	0.3	0.8	546.0	0.0

Table 16 Original data received from Search Light, and calculated amount of proteins in the samples. (Part 10)

		Placenta ng/mg	***Chorion ng/mg***	***TRAM ng/mg***	***Fresh AM ng/mg***	***SL ng/mg***
Angiogenesis	hFGFb	230.27	9.85	2.92	59.60	35.57
	hVEGF	15.09	15.14	9.77	14.74	20.91
	hHGF	74319.05	585.67	818.83	4808.70	3160.95
	hIGFBP1	5977.08	19160.33	118.18	480.77	3840.53
	hTIMP2	1990.30	267.54	102.57	169.64	152.22
	hTIMP1	631.42	476.68	167.07	217.15	270.71
Biomarker	hLeptin	40.12	30.71	7.61	55.29	43.51
	hLIF	0.71	0.57	0.08	1.64	1.06
	hProlactin	16.61	16.81	0.26	0.39	0.74
	hOPG	1.37	7.24	0.28	0.44	0.23
	hFibronectin	847852.53	382535.16	59658.39	225412.85	1967047.55
	hPEDF	1178.45	2481.08	0.00	1142.15	2021.15
Cell adhesion	hPselectin	0.41	0.18	5.06	10.81	3.47
	hLSelectin	421.35	162.08	110.03	88.66	95.94
	hICAM1	3181.18	385.94	80.02	93.09	187.60
	VCAM 1	2424.20	553.10	104.30	98.90	344.60
Cytokine	hIFNg	0.34	0.42	0.01	0.38	0.37
	hIL1a	0.02	0.02	0.01	0.04	0.02
	hIL1b	0.61	0.74	0.21	0.27	0.21
	hIL1ra	37.43	54.49	33.45	1206.11	612.64
	hIL2	0.33	0.34	0.05	0.28	0.33
	hIL3	0.00	0.00	0.00	0.00	0.00
	hIL4	0.03	0.02	0.10	0.09	0.15
	hIL5	0.16	0.20	0.14	0.14	0.28
	hIL7	0.00	0.01	0.00	0.00	0.00
	hIL9	0.00	0.91	0.00	0.45	0.74
	hIL10	0.03	0.01	0.01	0.00	0.00
	hTNFa	0.88	0.58	0.00	2.15	0.64
Growth factor	hEGF	0.41	0.18	5.06	10.81	3.47
	hPLGF	0.90	0.00	0.05	0.05	0.01
	hTGFB1	1181.18	106.09	74.13	49.71	76.82
	hTGFB2	119.32	32.29	11.62	8.66	20.30
	hHGH	158.52	18.41	3.79	4.63	8.33
	hTGFa	1.87	1.15	0.90	2.69	2.38
Metalloprotease	hTIMP1	631.42	476.68	167.07	217.15	270.71
	hTIMP2	1990.30	267.54	102.57	169.64	152.22
	hMMP2	3104.43	2627.80	209.12	389.93	874.02
	hMMP8	568.09	87.46	1.04	6.05	22.12
Neurotrophic factors	hCNTF	13.58	13.23	4.00	10.77	16.41
	hNT3	0.24	0.06	0.03	0.02	0.02
	hBNGF	0.40	0.12	0.04	0.02	0.04
	hBDNF	12.17	14.84	16.48	15.94	15.94

Table 17 SearchLight results. Search light results, protein expression in five tested samples. Soluble proteins were extracted from samples in triplicate and array was carried out in duplicate using SearchLight immunoassay technology (Aushton Biosystems, USA).

		hFGFb ng/mg	***hVEGF ng/mg***	***hHGF ng/mg***	***hIGFBP1 ng/mg***	***hTIMP2 ng/mg***	***hTIMP1 ng/mg***
AM 46	Placenta	230.27	15.09	74319.05	5977.08	1990.30	631.42
AM 46	Chorion	9.85	15.14	585.67	19160.33	267.54	476.68
AM 46	TRAM	2.92	9.77	818.83	118.18	102.57	167.07
AM 46	Fresh	59.60	14.74	4808.70	480.77	169.64	217.15
AM 46	SL	35.57	20.91	3160.95	3840.53	152.22	270.71
AM 72	Spongy layer washed	57.89	28.16	5351.29	101314.05	904.75	4693.35
AM 72	SL Unwashed	12.72	11.37	746.86	95093.74	509.18	3724.34
AM 72	SL Bloody	9.32	15.61	690.70	28301.46	331.99	547.52
AM 72	Foetal Blood	4.08	9.74	149.62	2480.07	322.54	1014.10
AM 55	1st wash of AM	83.28	199.71	37450.00	315451.50	15560.30	92904.00

Table 18 Angiogenic factors.
Angiogenic factors in tested samples.

		hLeptin ng/mg	***hLIF ng/mg***	***hProlactin ng/mg***	***hOPG ng/mg***	***hFibronectin ng/mg***	***hPEDF ng/mg***
AM 46	Placenta	40.12	0.71	16.61	1.37	847852.53	1178.45
AM 46	Chorion	30.71	0.57	16.81	7.24	382535.16	2481.08
AM 46	TRAM	7.61	0.08	0.26	0.28	59658.39	0.00
AM 46	Fresh	55.29	1.64	0.39	0.44	225412.85	1142.15
AM 46	SL	43.51	1.06	0.74	0.23	1967047.55	2021.15
AM 72	Spongy layer washed	122.39	3.20	154.10	4.22	3709483.14	16605.08
AM 72	SL Unwashed	39.79	1.04	424.70	6.99	928186.50	9380.77
AM 72	SL Bloody	35.68	1.06	2.56	1.57	2146548.03	2444.54
AM 72	Foetal Blood	87.54	6.38	83.03	0.61	116592.74	10961.86
AM 55	1st wash of AM	894.40	24.09	2261.00	12.39	26568780.00	502673.50

Table 19 Biomarkers tested in sent samples.
The amount of biomarkers in samples. Shows difference in ng/mg in 10 tested samples.

		hPselectin ng/mg	***hLSelectin ng/mg***	***hICAM1 ng/mg***	***VCAM 1 ng/mg***
AM 46	Placenta	0.40755	421.35405	3181.1832	2424.20
AM 46	Chorion	0.18224	162.0797	385.94412	553.10
AM 46	TRAM	5.0622	110.0286	80.02071	104.30
AM 46	Fresh	10.81405	88.66325	93.09235	98.90
AM 46	SL	3.47425	95.9374	187.59845	344.60
AM 72	Spongy layer washed	10.77494	589.44322	711.339	1367.80
AM 72	SL Unwashed	0.44516	407.39766	237.60322	583.50
AM 72	SL Bloody	2.58984	144.84558	113.03622	486.00
AM 72	Foetal Blood	2.69344	4155.1936	540.227	5753.20
AM 55	1st wash of AM	53.592	6611.087	4016.04	36846670.00

Table 20 Cell adhesion proteins in tested samples.

		hIL5	*hTNFa*	*hIL1a*	*hIL2*	*hIFNg*	*hIL1ra*	*hIL10*	*hIL9*	*hIL7*	*hIL4*	*hIL3*	*hIL1b*
AM 46	***Placenta***	0.1599	0.8775	0.01999	0.3276	0.3393	37.43415	0.03315	0	0	0.03185	0	0.60645
AM 46	***Chorion***	0.19966	0.5829	0.0213	0.34438	0.41942	54.48507	0.01139	0.9112	0.0067	0.02211	0	0.74102
AM 46	***TRAM***	0.13728	0	0.01488	0.05148	0.00924	33.45474	0.00594	0	0	0.10362	0	0.2145
AM 46	***Fresh***	0.1443	2.1515	0.03574	0.27755	0.3783	1206.114	0.0039	0.4485	0	0.09425	0	0.26845
Am 46	***SL***	0.27625	0.6435	0.02474	0.3302	0.3653	612.638	0.0039	0.741	0	0.15015	0	0.21255
AM 72	***Spongy layer washed***	0.25862	5.6682	0.04904	1.07736	1.20064	1442.8718	0.02412	0	0.0134	0.15544	0	1.09478
AM 72	***SL Unwashed***	0.1085	0.4092	0.01785	0.3627	0.4185	142.972	0.02108	0	0.0062	0.01178	0	0.67642
AM 72	***SL Bloody***	0.09174	1.452	0.02055	0.38808	0.35574	145.8204	0.0396	0.0858	0.0066	0	0	0.51084
AM 72	***Foetal Blood***	0.20824	6.7602	0.07231	1.35774	0.49134	72.0936	0.05054	37.7302	0.4826	1.59182	4.7538	0.47006
AM 55	***1st wash of AM***	3.283	34.3	0.02899	4.557	5.516	8915.62	0.546	4.41	1.19	5.25	4.76	6.79

Table 21 Cytokine proteins detected in tested samples. Cytokine proteins were tested in the samples, results present in ng/mg.

	hEGF– human epidermal growth factor	***hPLGF – human placenta like growth factor***	***hTGFβ1– human transforming growth factor β1***	***hTGFβ2– human transforming growth factor β2***	***hHGH – human growth hormone***	***hTGFα – human transforming growth factor α***
Placenta	0.4	0.9	1181.18	119.32	158.52	1.87
Chorion	0.2	0	106.09	32.29	18.41	1.15
TRAM	5.1	0.052	74.13	11.62	3.79	0.90
Fresh AM	10.8	0.052	49.71	8.66	4.63	2.69
SL	3.5	0.013	76.82	20.30	8.33	2.38
Spongy layer washed	10.8	0	201.23	69.40	20.45	5.26
SL Unwashed	0.4	0.335	92.05	23.63	23.10	1.41
SL Bloody	2.6	0	258.38	44.78	9.88	1.49
Foetal Blood	2.7	0.144	55.0	32.62	17.28	2.07
1st wash of AM	53.6	3.01	893.66	307.26	293.15	82.68

Table 22 Growth factors in tested samples.
Amount of growth factors in tested samples. Table shows different amount of EGF, PLGF, TGFβ1, TGFβ2, HGH, and TGFα in 10 analysed samples.

	hTIMP1– human Tissue Inhibitor of Metalloprotease	***hTIMP2 – human Tissue Inhibitor of Metalloprotease***	***hMMP2 – human Matrix Metalloprotease 2***	***hMMP8 – human Matrix Metalloprotease 8***
Placenta	631.42	1990.3	3104.43	568.09
Chorion	476.68	267.54	2627.80	87.46
TRAM	167.07	102.57	209.12	1.04
Fresh	217.15	169.64	389.93	6.05
SL	270.71	152.22	874.02	22.12
Spongy layer washed	4693.35	904.75	12651.54	163.21
SL Unwashed	3724.34	509.18	3438.25	182.78
SL Bloody	547.52	331.99	5473.98	228.52
Foetal Blood	1014.10	322.54	1953.06	1298.57
1st wash of AM	92904	15560.3	328076	1049.64

Table 23 Metalloproteinase in tested samples.
Level of metalloproteinases has been tested in 10 samples. Figures represent amount of proteins in ng/mg. Comparison made between samples.

	hCNTF – human Cilliary Neurotrophic Factor	***hNT3 – human Neurotrophin 3***	***hNGFβ – human Nerve Growth Factor β***	***hBDNF – human Brain Derived Neurotrophic Factor***
Placenta	13.58	0.24	0.40	12.17
Chorion	13.23	0.06	0.12	14.84
TRAM	4.00	0.04	0.04	16.48
Fresh	10.77	0.02	0.02	15.94
SL	16.42	0.02	0.04	15.94
Spongy layer washed	25.47	0.07	0.09	32.86
SL Unwashed	10.25	0.07	0.10	15.21
SL Bloody	11.52	0.09	0.12	16.19
Foetal Blood	12.85	0.37	0.84	9.32
1st wash of AM	346.16	3.46	10.47	171.67

Table 24 Neurotrophic factors in tested samples.
The amount of neurotrophic factors in 10 tested samples. Show amount and difference in ng/mg.

	Protein name	***Acc No***	***Alternative name***	***Subcellular location***	***Function/involved /related to AM***	***Biological process***	***Mr (Nominal mass)***	***peps***
Profilin-1	PROF1_HUMAN	P07737	Epididymis tissue protein Li184a/Profilin-1	cytoplasm/ cytoskeleton	binds to actin and affects the structure of the cytoskeleton	cell death/cellular response to growth factor stimulus	15045	47
Tubulin polymerization-promoting protein family member 3	TPP3_HUMAN	O94811	25kDa brain-specific protein	cytoplasm/ cytoskeleton/ nucleus	may play role in the polymerization of tubulin into microtubules	microtubulin bundle formation	23694	210
SH3 domain-binding glutamic acid-rich-like protein 3	SH3L3_HUMAN	Q9H299	SH3 domain-binding protein 1	cytoplasm/ nucleus	could act as a modulator of glutaredoxin biological activity	cell redox homeostasis/oxidation-reduction process	10438	90
Tubulin polymerization-promoting protein family member 3	TPPP3_HUMAN	Q9BW30		cytoplasm/ cytoskeleton	binds tubulin and has microtubule bounding activity/ may play role in cell proliferation and mitosis	microtubulin bundle formation	18985	170

Ubiquitin-40S ribosomal protein S27a	RS27A_HUMAN	P62979	Ubiquitin carboxyl extension protein 80	cytoplasm/ nucleus	covalently attached to another protein, or free (unanchored). Involved in DNA repaired and lysosomal degradation, when free has a distinct role such as inactivation of protein kinases and signalling	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest/T cell receptor signalling pathway/I-kappa B kinase/NF-kappaB signalling	17965	150
Actin, aortic smooth muscle	ACTA_HUMAN	P62736	Alpha-actin-2/Cell growth-inhibiting gene 46 protein		Acting are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells		42009	370
Protein S100-A6	S10A6_HUMAN	P06703	Calcyclin/Growth factor-include protein 2A9	cell membrane/ cytoplasm membrane/ nucleus	calcium sensor and modulator/may function by interacting with other proteins, such as TPR-containing proteins	axonogenesis/positive regulation of fibroblast proliferation/signal transduction	10180	90

SH3 domain-binding glutamic acid-rich-like protein	SH3L1_HUMAN	O75368	SH3 domain-binding glutamic acid-rich-like protein	cytoplasm/ **extracellular** space/extracellular vesicular exosome/ nucleus			12774	110
Thioredoxi	THIO_HUMAN	P10599	ATL-derived factor	cytoplasm/ nucleus/ **secreted**	oxidoreductase activity, cell proliferation	electron transport/transcription	11737	100
SH3 domain-binding glutamic acid	SH3 domain-binding glutamic acid-rich-like protein 3	Q3ZCL8	SH3BGRL3	cytoplasm/ nucleus	could act as a modulator of glutaredoxin biological activity	cell redox homeostasis/oxidation-reduction process	10431	68
Peptidyl-prolyl cis-trans isomerise A	PPIA_HUMAN	P62937	Peptidyl-prolyl cis-trans isomerise A/Pplase A EC5.2.1.8/ Cyclophilin A/Rotamase A	cytoplasm/ **Secreted**	accelerate the folding of proteins/ interacts with HIV-1	host-virus interaction/leukocyte migration	18001	25
Phosphatidyl ethanolamine-binding protein 1	PEBP1_HUMAN	P30086	PEBP-1/HCNPpp/Neuropolypeptide h3	Cytoplasm	binds ATP/may be involved in the function of the presynaptic cholinergic neurons of the central nervous system	negative regulation of endopeptidase activity/ATP binding	21044	50

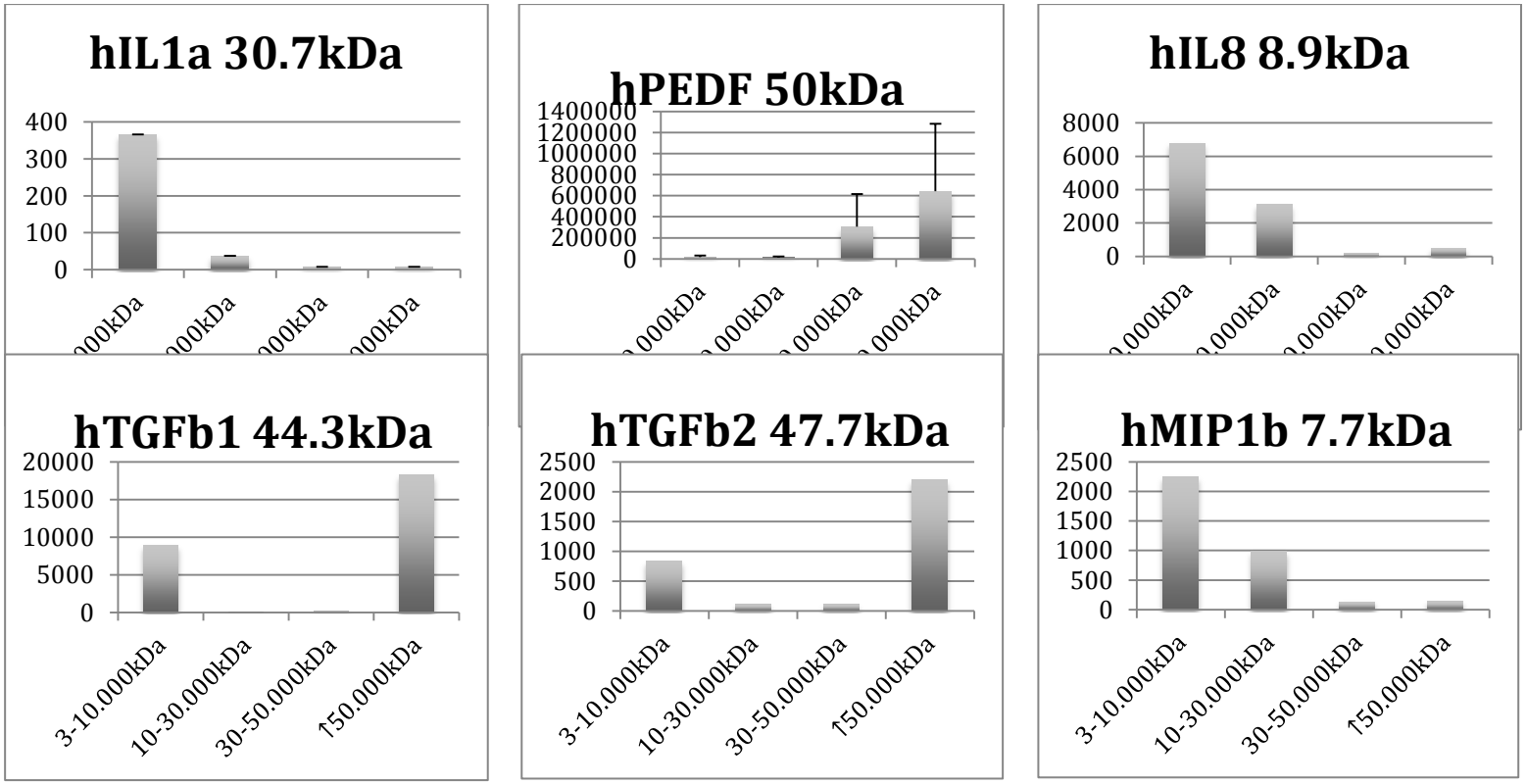
Tubulin polymerization-promoting protein family member 3		Q9BW30	TPPP3_HUMAN /HGNC/brain specific protein/TPPP/p20	cytoplasm/ cytoskeleton/ microtubule	binds tubulin and has microtubule bounding activity/ may play role in cell proliferation and mitosis	microtubule binding formation	18974	170
Thymosin beta-4-like protein 2		P18758	Thymosin beta 4Xen	cytoplasm/ cytoskeleton	important role in organisation of the cytoskeleton	actin cytoskeleton organization	5060	34
Thymosin beta-4		P62328	Seraspenide	Cytoplasm/ Cytoskeleton	organization of the cytoskeleton/seraspenide inhibits the entry of haematopoietic stem cells into the S-phase	actin cytoskeleton organization	5050	26
Transgelin-2		P37802	Epididymis tissue protein Li7e/SM22-alpha homolog		muscle organ development		22377	17

Table 25 Identified proteins in SL sample.

Function	***Factors***	***FRAM (ng/mg)***	***SL (ng/mg)***	
Angiogenesis	hAng 2	0.005-/+0.005	0.355+/-0.222	p=0.741
	hFGFbasic	0.609-/+0.363	5.375+/-1.302*	p=0.021 - stats.significant
	hHGF	34.004-/+14.493	933.214+/-374.92*	p=0.021 - stats.significant
	hKGF	0.106-/+0.060	5.807+/-3.576*	p=0.021 - stats.significant
	hTIMP 1	97.548-/+47.431	515.589+/-196.495	p=0.083
	hTIMP 2	29.566-/+13.229	610.743+/-362.522*	p=0.021 - stats.significant
	hVEGF	0.006-/+0.003	0.254+/-0.047*	p=0.047 - stats.significant
Biomarker	hFASL	0.003-/+0.000	0.080+/-0.015*	p=0.013 - stats.significant
	hFibrinonectin	262.972-/+147.568	120161.273+/-109287.844*	p=0.021 - stats.significant
	hPEDF	85.643-/+55.701	349.321+/-134.329*	p=0.043 - stats.significant
	hTRAIL	0.104-/+0.044	0.567+/-0.150*	p=0.021 - stats.significant
	hTSP 1	1440.505-/+659.289	564.021+/-206.844	p=0.248
Cell adhesion	hICAM 1	8.830-/+3.573	201.414+/-97.651*	p=0.021 - stats.significant
	hICAM 3	2.149-/+0.911	271.526+/-111.880*	p=0.021 - stats.significant
	hVCAM 1	18.405-/+9.258	1394.851+/-607.772*	p=0.021 - stats.significant
	hE-Selectin	0.415-/+0.160	11.262+/-3.564*	p=0.021 - stats.significant
Chemokine	hRANTES	0.066-/+0.031	1.462+/-1.115*	p=0.021 - stats.significant
	hMIP 1a	0.078-/+0.032	0.647+/-0.228	p=0.191
	hMIP 1B	0.023-/+0.011	0.298+/-0.101*	p=0.021 - stats.significant
	hMIF	1762.011-/+713.587	1697.356+/-156.325	p=1.0
Cytokine	hIFNy	0.001-/+0.000	0.014+/-0.007	p=0.739
	hIL 1a	0.047-/+0.021	0.218+/-0.056*	p=0.021 - stats.significant
	hIL 1b	0.001-/+0.000	0.016+/-0.004*	p=0.018 - stats.significant
	hIL 1ra	229.968-/+100.232	337.314+/-78.737	p=0.773
	hIL 6	0.018-/+0.008	0.662+/-0.140*	p=0.021 - stats.significant
	hIL 8	0.116-/+0.058	2.487+/-0.915*	p=0.021 - stats.significant

	hIL 10	0.000-/+0.000	0.021+/-0.004	p=1.0
	hTNFa	0.006-/+0.002	0.122+/-0.023*	p=0.047 - stats.significant
Growth factor	hEGF	0.946-/+0.429	1.27+/-0.934	p=0.773
	hHBEGF	0.000-/+0.000	0.096+/-0.018	p=1.0
	hHGH	0.811-/+0.444	61.928+/-29.598*	p=0.021 - stats.significant
	hSCF	0.173-/+0.069	1.140+/-0.342	p=0.021 - stats.significant
	hTGFa	0.036-/+0.024	0.132+/-0.32	p=0.059
	hTGFB 1	2.753-/+1.436	187.062+/-79.810*	p=0.021 - stats.significant
	hTGFB 2	0.469-/+0.212	39.829+/-16.656*	p=0.021 - stats.significant
Metalloprotease	hMMP 1	0.285-/+0.201	6.985+/-5.804	p=0.741
	hMMP 2	7.705-/+3.3035	1469.26+/-1116.238*	p=0.021 stats.significant
	hMMP 3	0.194-/+0.080	10.904+/-4.225*	p=0.021 stats.significant
	hMMP 7	0.248-/+0.103	3.342+/-0.239*	p=0.021 stats.significant
	hMMP 8	0.245-/+0.117	191.489+/-79.108*	p=0.021 stats.significant
	hMMP 9	1.308-/+0.523	7897.577+/-6408.404*	p=0.021 stats.significant
	hMMP 10	0.820-/+0.326	121.233+/-62.914*	p=0.021 stats.significant
	hMMP 13	0.000-/+0.000	0.506+/-0.094	p=1.0
Neurotrophic factor	hb-NGF	0.003-/+0.001	0.022+/-0.005	p=0.191
	hBDNF	1.493-/+0.587	37.215+/-14.886*	p=0.021 - stats.significant
	hCNTF	0.034-/+0.013	0.233+/-0.010	p=0.442
	hGDNF	0.016-/+0.019	0.325+/-0.192	p=0.166
	hNT 3	0.000-/+0.000	0.022+/-0.015	p=0.321

Table 28 SearchLight results comparison between FRAM and SL statistically calculated. This table shows amount of the tested factors in the samples with calculated statistical results. Majority factors have statistically significant variations.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2000
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150.000kDa
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150.000kDa
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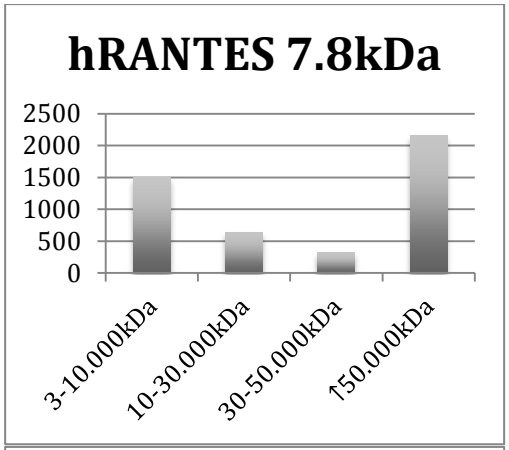
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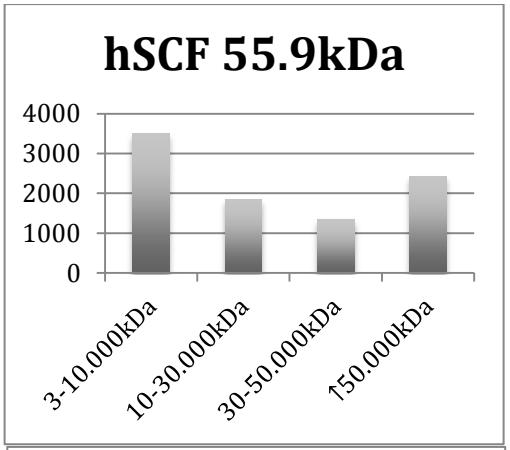
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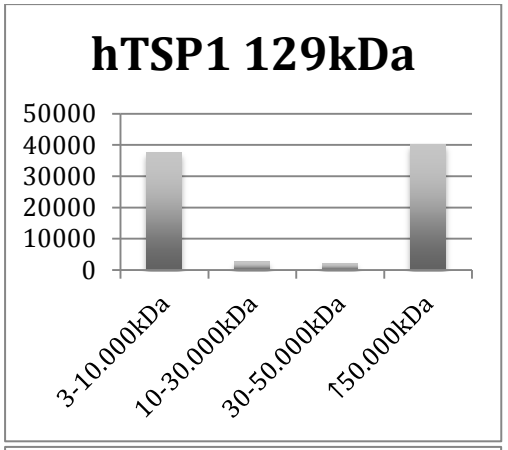
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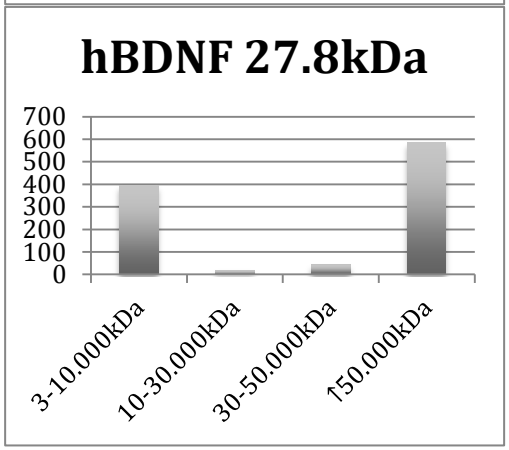
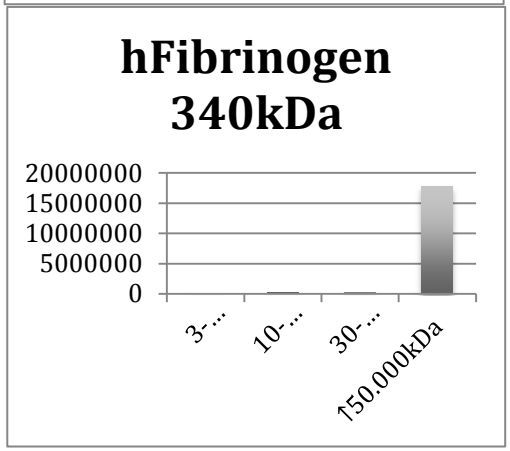
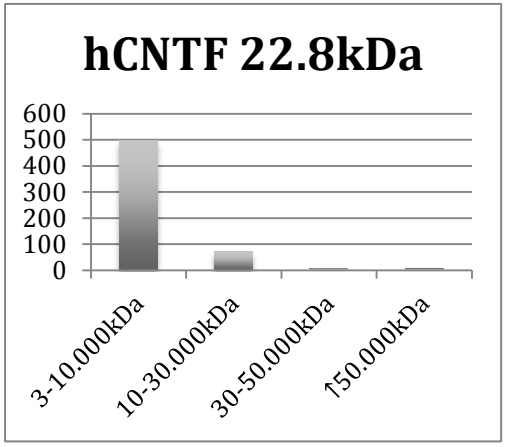
30-50.000kDa
↑50.000kDa


hBDNF 27.8kDa
700
600
500
400
300
200
100
0
3-10.000kDa
10-30.000kDa
30-50.000kDa
↑50.000kDa


hFibrinogen 340kDa
20000000
15000000
10000000
5000000
0
3-…
10-…
30-…
↑50.000kDa


hCNTF 22.8kDa
600
500
400
300
200
100
0
3-10.000kDa
10-30.000kDa
30-50.000kDa
↑50.000kDa

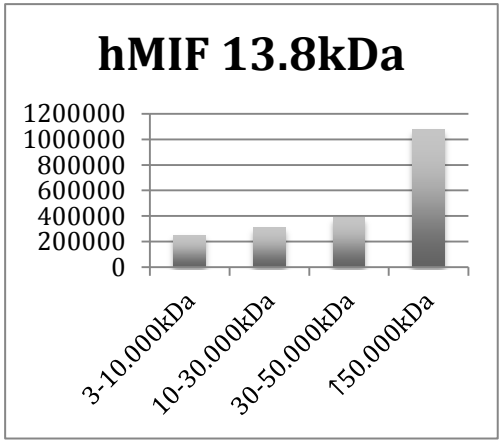
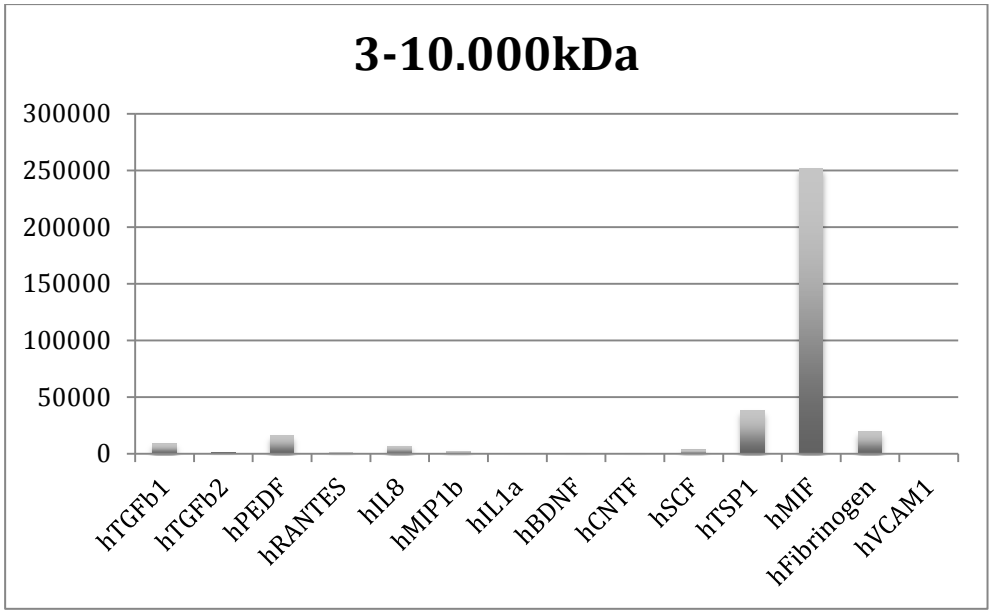
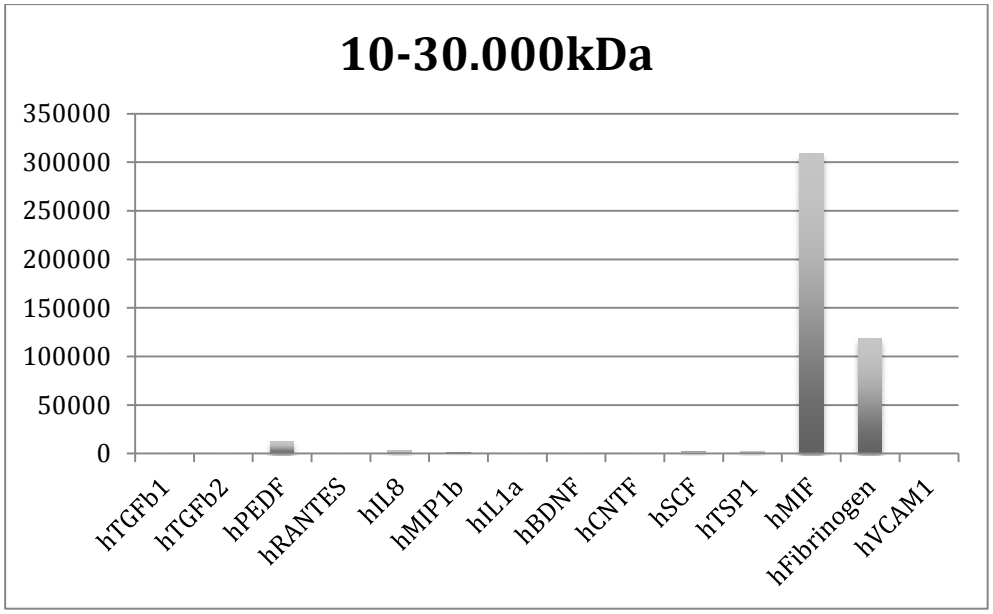
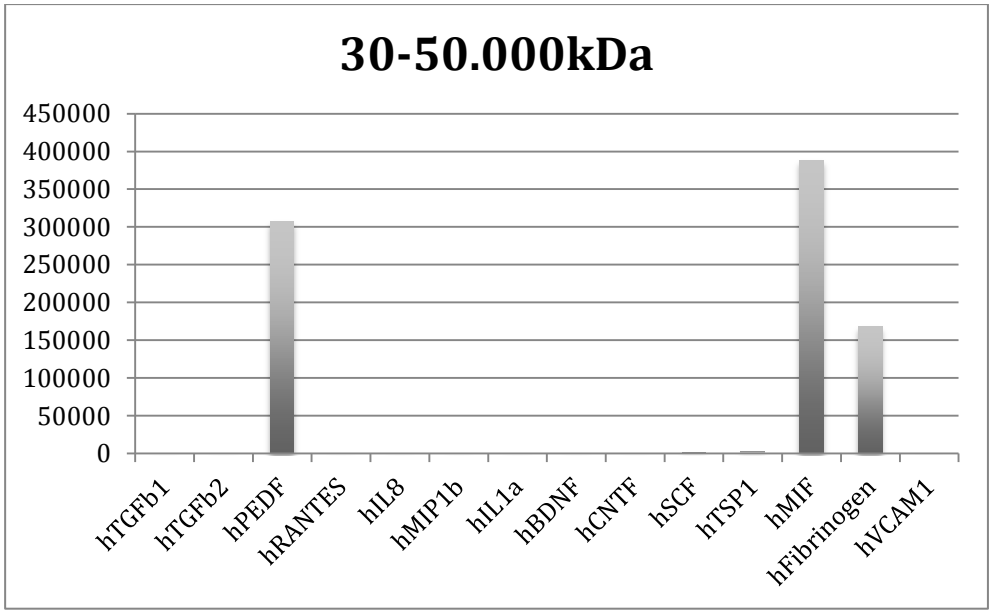


Table 29. Abundance of proteins in SL fractions. This table shows the amount of present factor in the fractions, where can be seen present of factors in fractions with "wrong" molecular weight.


3-10.000kDa
300000
250000
200000
150000
100000
50000
0
hTGFb1
hTGFb2
hPEDF
hRANTES
hIL8
hMIP1b
hIL1a
hBDNF
hCNTF
hSCF
hTSP1
hMIF
hFibrinogen
hVCAM1


10-30.000kDa
350000
300000
250000
200000
150000
100000
50000
0
hTGFb1
hTGFb2
hPEDF
hRANTES
hIL8
hMIP1b
hIL1a
hBDNF
hCNTF
hSCF
hTSP1
hMIF
hFibrinogen
hVCAM1


30-50.000kDa
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
hTGFb1
hTGFb2
hPEDF
hRANTES
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hSCF
hTSP1
hMIF
hFibrinogen
hVCAM1

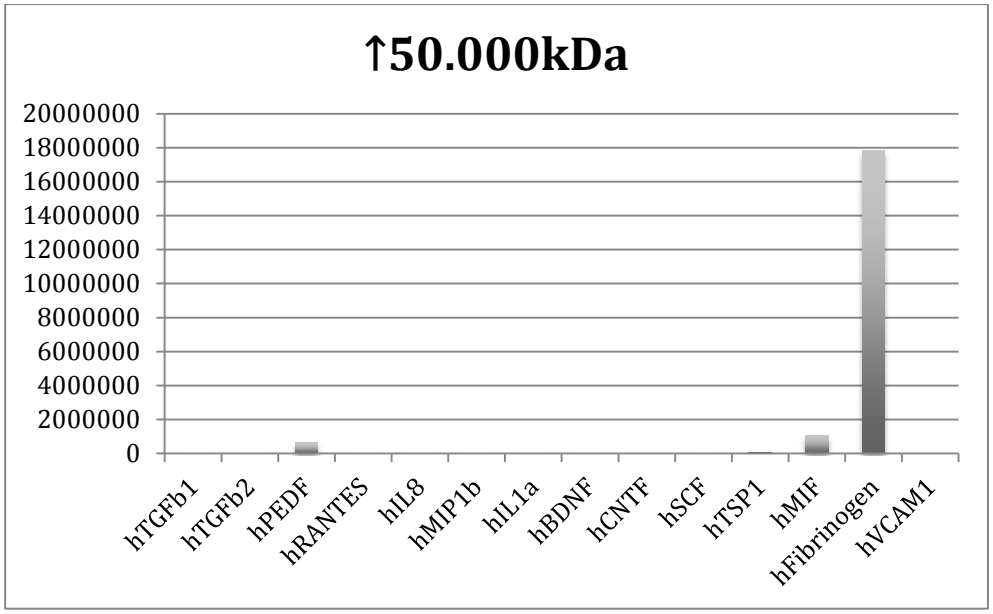

Table 30. Comparison of the amount of proteins in the different SL fractions. Four different SL fractions with molecular mass of proteins 3 000-10 000kDa; 10 000-30 000kDa; 30 000-50 000kDa; and more that 50 000kDa, were tested on the amount of proteins in each fraction

		S. aureus 10^6 CFU/mL MIC				S. epidermidis 10^6 CFU/mL MIC				Diptheroid 10^6 CFU/mL MIC				Moraxella 10^6 CFU/mL MIC			
Wells	**%**	**SL177**	**SL180**	**SLPo^{16}**	**Gent**	**SL177**	**SL180**	**SLPo^{16}**	**Gent**	**SL177**	**SL180**	**SLPo^{16}**	**Gent**	**SL177**	**SL180**	**SLPo^{16}**	**Gent**
1	**100**	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	**50**	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
3	**25**	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-
4	**12.5**	+	+	-	-	+	-	+	-	+	+	+	-	-	+	-	-
5	**6.25**	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-
6	**3.13**	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-
7	**1.56**	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-
8	**0.78**	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-
9	**0.39**	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
10	**0.20**	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
11	**0.10**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	**-**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 32. MIC of SL samples against gram (-) and gram (+) bacteria. Minimum Inhibitory Concentration (MIC) of SL samples against a panel of gram negative and gram positive bacteria. (+) showed growth of microorganism, (-) no growth of microorganisms, well 11 positive control, well 12 negative control. The results are shown as average values from three separate experiments. Gent – gentamicin was used as a reference.

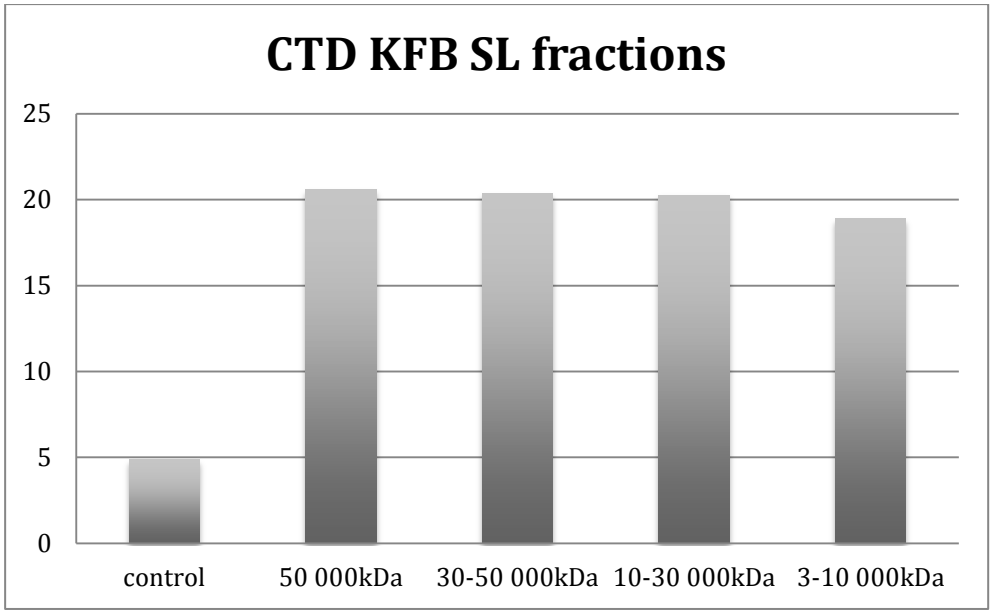

Figure 33. CTD KFB SL fractions. SL fractions induce cytotoxic activity of KFB compare to control sample. In all protein weight range.

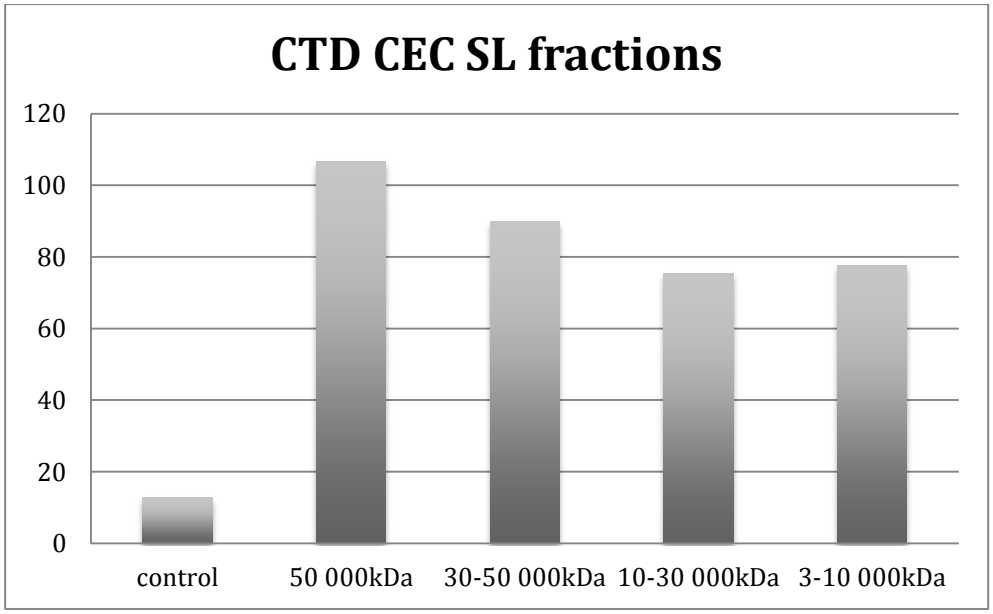

Figure 34. CTD CEC SL fractions. SL fractions induce cytotoxic activity of CEC compare to control plate in all proteins weight range.

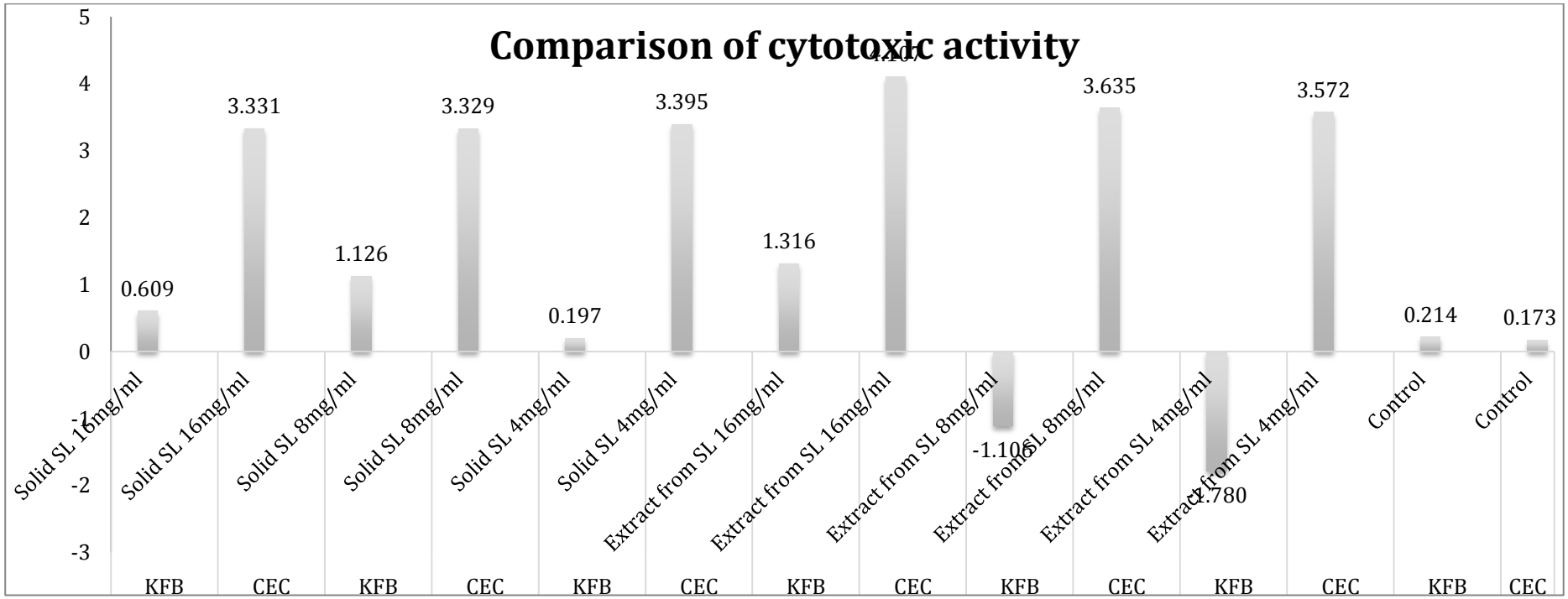

Figure 35. Comparison of cytotoxic activity. Figure compared the cytotoxic activity between corneal epithelial cells and keratocytes treated with extract of SL in 4 different concentrations.Comparison made between two different cells type and control plates for both cells type.

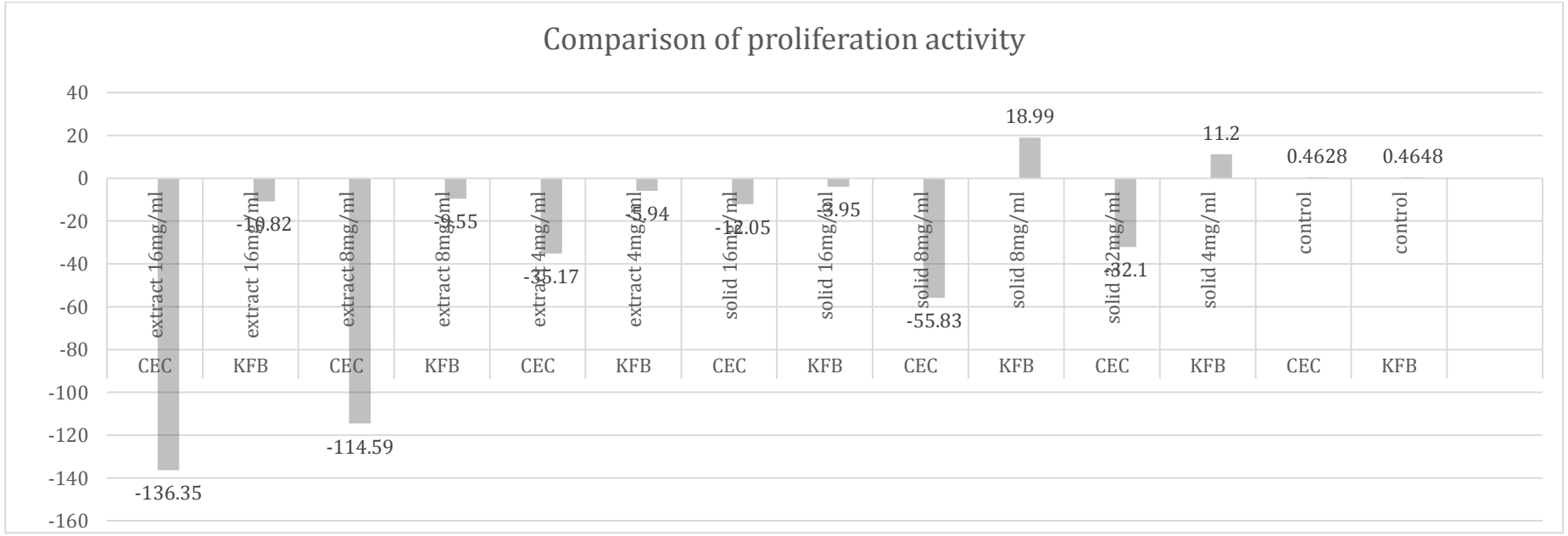

Figure 36. For the second test SL samples were fractionated (see method above) and results shown

fractionated (see method above) and results show

Figure 36. Comparison of proliferation activity. Figure compared the proliferation activity between corneal epithelial cells (CEC) and keratocytes (KFB) treated with extract of SL in 4 different concentrations.Comparison made between two different cells type and control plates for both cells type

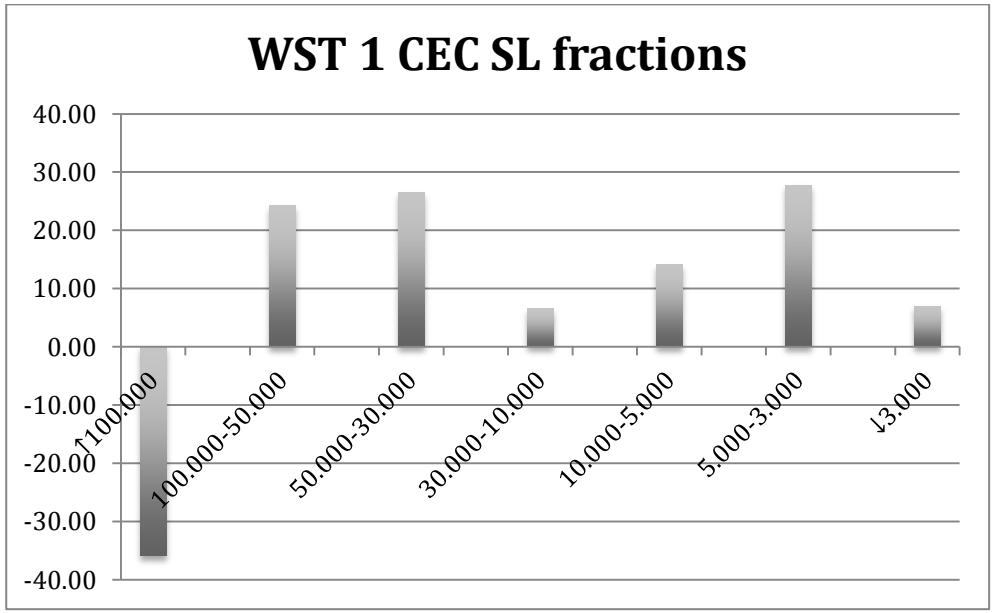

Figure 37. WST 1 CEC SL fractions. After SL fractionation, samples were tested on CEC for proliferation activity of each fraction. Only one fraction appears to have negative proliferation effect.

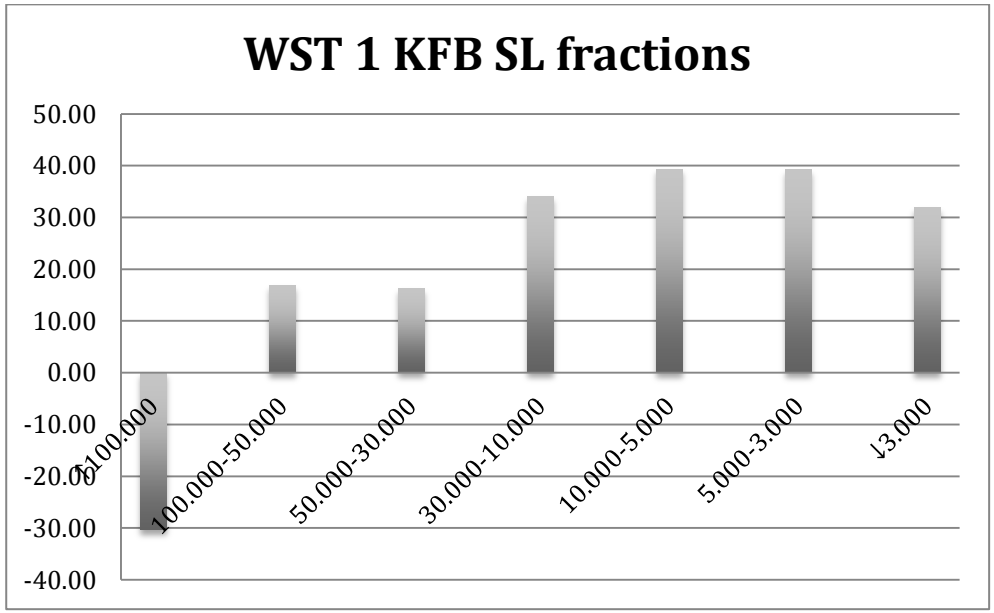

Figure 38. WST 1 KFB SL fractions. After fractionation, samples were tested on KFBs for proliferation activity of each sample. First fraction with molecular mass of proteins with more than 100 000 kDa, was negative.

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