INVESTIGATION OF INFLAMMATORY RESPONSES FOLLOWING THE USE OF NOVEL ANISOTROPIC HYDROGEL TISSUE EXPANDER IN THE SKIN OF MAXILLOFACIAL REGION

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF DENTAL SCIENCE

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2020

UNIVERSITY OF MALAYA

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ABSTRACT

Background: The purpose of tissue expander is to grow healthy supplementary tissue under the controlled of mechanical force exerted by the tissue expander. In the field of reconstructive and plastic surgery, the use of soft tissue expanders are important for growing healthy supplementary tissue. However, the molecular mechanism associated with the inflammatory response associated with the force generated from the use of tissue expanders is still not characterized. Among the many types of tissue expanders, anisotropic type of hydrogel tissue expander was chosen due to its ability to produce controlled incremental inflation on the tissue that enhance cellular adaptation and the ability of the cells to resist mechanical injury. Objective: To determine whether anisotropic hydrogel tissue expander promotes subclinical inflammatory response by triggering the secretion of pro-inflammatory cytokines. Material and Methods: Seven, 8 weeks old Sprague Dawley rats (300g ± 50g) were used as models. The rats were randomly divided into two groups: control group (n=3) and expanded group (n=4). The expanders were implanted subcutaneously at the forehead region for 28 days and surgically removed. The rats were sacrificed and skin samples were harvested and stored in 10% formaldehyde, fixed in formalin, and embedded in paraffin wax for histological investigation. Hematoxylin and eosin staining was performed to detect histological changes between the two groups and to detect the presence of an inflammatory response in the expanded samples. Moreover, the expression level of pro-inflammatory cytokines IL-1, TNF-α, IL-8, and IL-6 were determined by immunohistochemistry. Immune positivity was detected using secondary antibody conjugated with horseradish peroxidase (HRP) after staining with diaminobenzidine tetrahydrochloride (DAB). Results: Histological analysis showed no inflammatory response was present in the expanded tissue. There was no significant difference in cytokine production level between the expanded and control samples ($p = 0.071$).

However, there was very strong expression of IL-1 in the expanded tissue as 83 % of the cells were immune-positive compared to the controls. In contrast, there was no significant difference in IL-6, IL-8, and TNF-α production. A negative non-significant correlation between IL-1 immune-positive cells and the inflammatory cells in the expanded tissue was observed with a Spearman's correlation coefficient of r_s -0.500, p=0.667. Conclusion: The *in vivo* experiment revealed a high biocompatibility of the anisotropic self-inflating hydrogel tissue expander and no significant immunological or inflammatory reaction were observed between the control and tissue expanded groups.

Keywords: Tissue expansion, Anisotropic hydrogel, Skin, Cytokines, Inflammation.

ABSTRAK

Latar belakang kajian: Tujuan utama penggunaan alat pengembang tisu adalah untuk membina tisu tambahan yang sihat di bawah kawalan kuasa mekanikal yang dikenakan oleh alat pengembang tisu. Dalam bidang rekonstruksi dan pembedahan plastik, penggunaan alat pengembang tisu lembut merupakan satu kaedah yang penting untuk mendapatkan pertumbuhan tisu baru. Berikutan dengan penggunaan alat pengembang tisu, tisu di sekitar alat pengembang tisu tersebut akan bertindakbalas melalui proses keradangan. Walaupun kajian tentang penggunaan alat pengembang tisu telah banyak dijalankan, tidak banyak yang diketahui tentang proses atau mekanisma keradangan yang berlaku berikutan penggunaan alat pengembang tisu. Bagi kajian ini, alat pengembang tisu jenis hidrogel anisotropik dipilih daripada pelbagai jenis alat pengembang tisu disebabkan kemampuan alat tersebut untuk menghasikan pertambahan inflasi pada tisu secara terkawal disamping dapat meningkatkan penyesuaian sel terhadap alat pengembang tersebut dan meningkatkan juga kemampuan sel untuk menentang kecederaan mekanikal. Objektif: Untuk menentukan samada pengembang tisu jenis hidrogel anisotropik boleh mencetuskan tindak balas keradangan subklinikal melalui pembebasan sitokin 'pro-inflammatory'. Bahan dan Kaedah: Tujuh ekor tikus 'Sprague dawley' (300g ± 50g) berusia 8 minggu digunakan sebagai model untuk kajian melibatkan penggunaan pengembang tisu. Tikus ini dibahagikan secara rawak kepada dua kumpulan: kumpulan control (n=3) dan kumpulan tisu pengembang (n=4). Bahan pengembang tisu diimplan secara subkutaneus di bahagian dahi selama 28 hari sebelum dibedah keluar. Tikus-tikus ini dikorbankan dan sampel kulit diambil dan dimasukkan ke dalam formaldehid 10%, diawetkan dalam formalin dan diserap ke dalam lilin parafin untuk kajian histologi. Kajian histologi melibatkan penggunaan penstainan 'hematoxylin' dan 'eosin' untuk mengesan perubahan histologi antara kumpulan kawalan dan kumpulan pengembang tisu. Disamping itu, kehadiran tindak balas

keradangan juga turut dikaji diantara kedua-dua kumpulan tersebut. Tambahan lagi, tahap ekspresi sitokin 'pro-inflammatory' IL-1, TNF-α, IL-8 dan IL-6 turut ditentukan melalui teknik imunohistokimia. Imun positif akan dikesan menggunakan antibody sekunder berkonjugat dengan 'horseradish peroxidase' (HRP) setelah diwarnai dengan diaminobenzidine tetrahydrochloride (DAB). Keputusan: Analisis histologi menunjukkan tidak terdapat tindak balas keradangan dengan penggunaan alat pengembang tisu. Tambahan juga, tidak terdapat perbezaan signifikan terhadap tahap penghasilan sitokin antara sampel yang menggunakan pengembang tisu dan sampel kawalan (p=0.071). Walaubagaimanapun, terdapat ekspresi yang kuat untuk IL-1 pada sampel yang menggunakan pengembang tisu dimana 83% daripada sel-sel sampel dalam kumpulan tersebut didapati adalah imun positif berbanding sampel dari kumpulan kawalan. Sebaliknya, tiada perbezaan signifikan bagi ekspresi IL-6, IL-8, dan penghasilan TNF-α. Keputusan dari kajian ini juga menunjukkan tiada korelasi negatif yang signifikan bagi diantara IL-I sel imun positif dan sel keradangan (r=-0.500, p=0.667) menggunakan korelasi koefisien Spearman. Kesimpulan: Kajian in vivo ini telah menunjukkan bahawa penggunaan pengembang tisu jenis anisotropik hidrogel pengembang sendiri mempunyai sifat biokompatibiliti yang tinggi dan tiada perbezaan imunologi atau tindak balas keradangan yang signifikan di antara kumpulan kawalan dengan kumpulan yang menggunakan pengembang tisu.

Kata kunci: Pengembang tisu, Hidrogel anisotropik, Kulit, Sitokin, Keradangan.

ACKNOWLEDGEMENTS

First and above all, I praise God, the Almighty for providing me the opportunity to finish my postgraduate study and granting me the capability to perform this work.

Undertaking this Masters degree in research has been a tough, yet life changing experience for me, and I could not have been able to finish this work without the support and the guidance that I received from many. First, I am gratefully indebted to my supervisors Dr. Siti Mazlipah Binti Ismail, Associate Professor Dr. Zamri Radzi, and Professor Dr. Mohammed Tariqur Rahman for their willingness to share their knowledge with me, critical comments, and providing me with continuous scientific suggestions throughout the preparation of my dissertation and manuscript.

I gratefully acknowledge the help and support of technical and laboratory staff at the Faculty of Dentistry, University of Malaya.

Last but not least, I would like to acknowledge the people who means the life to me, my husband Dr. Mohammed, for providing me the support and guidance through this period, his help and respect, without him it would be impossible for both of us to finish our postgraduate studies at the same time. To my sweetie daughter Jannat and my two lovely sons Amjad and Anas, every day I thought it is tough and I would stop, but once I see smiles on their faces and their unconditional love, it gave me support and energy to believe in myself, moreover, to be a role model for them. To my parents, my brother, and my grandmother, I do believe that every person had helped me is because of their blessings and prayers, and to my uncles and aunts for their support, although we are on the other side of the world, but they did not forget us.

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LIST OF SYMBOLS AND ABBREVIATION

IL	:	Interleukin
TNF-α	:	Tumor Necrosis Factor Alpha
WBC	:	White Blood Cells
MCP-1	:	Monocyte Chemoattractant Protein-1
STE	:	Soft Tissue Expander
CRP	:	C-Reactive Protein
H&E	:	Hematoxylin and Eosin
IHC	:	Immunohistochemical
DAB	:	3,39-Diaminobenzidine Tetrahydrochloride
PBS	:	Phosphate Buffer Saline
BSA	:	Bovine Serum Albumin
HRP	:	Horseradish Peroxidase
P(AMM-NVP)	:	Methyl-Methacrylate and N-Vinylpyrrolidone Copolymer
HCL	:	Hydrochloric Acid
L	:	Liter
μ	:	Micro
β	:	Beta
α	:	Alpha
pg/ml	:	Pico gram

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CHAPTER 1

INTRODUCTION

1.1 Background

The purpose of tissue expansion in reconstructive surgery is to grow healthy supplementary tissue under controlled mechanical tensile force for replacement of damaged tissue had been reported by various researchers (Asa'ad et al., 2016; Garner et al., 2017). Since the first utilization of soft tissue expansion in 1957 (Nuemann, 1957) it has become an alternative choice (John et al., 2015) to flaps and tissue grafts (Austad &Rose, 1982). Tissue expanders are specially designed devices to produce stress beneath localized tissue (Jamadi et al., 2017). Traditionally, balloon-type silicon expanders were used which expanded by being continuously refilled with air (Feng et al., 2016). This technique was first reported by Neumann in 1957, and twenty years later in the 1970s Radovan and Argenta improved on Neumann's expander model by filling the balloon with saline instead of air.

The expander was used to regenerate tissue for breast reconstruction after breast cancer surgery. As the technique advanced, new expander materials were developed (Egeland & Cederna, 2008; Fan & Wang, 2004) to customize the volume of expansion and to suit the size of different anatomical sites for reconstruction of a wide range of skin and soft tissue defects (Fan & Wang, 2004). These expanders were used in areas such as cranio-maxillofacial surgery (Barwinska et al., 2017; Philip, 1992), head and neck region (Handschel et al., 2013), and nasal reconstruction (Ramanathan, 2014), ophthalmology (Helmuth et al., 2005), breast reconstruction after mastectomy (Brooke et al., 2012), pediatric hypospadias (Lansdale et al., 2015), burn scar ablation (Fochtmann et al., 2013). Successful clinical application of soft tissue expander to

achieve skin closure in open large bone fractures was also reported (Formby et al. 2013).

Nevertheless, the successful application of the inflatable tissue expanders are not without complications (Huang et al., 2011). The larger the area to be expanded the higher the chance of complication, thus the location of expansion is a risk factor (Braun et al., 2016; Neta et al., 2015). Other complications range from mild to severe such as hematoma due to blood accumulation between the flap and the expander balloon, partial or complete necrosis of the tissue leading to cyanosis and hypoxia due to vigorous stretching from the device, rupture, ectropion, and migration of expander unit (Hurvitz et al., 2005; McCullough et al., 2017).

As a result, a self-inflating tissue expander that does not require external inflation was introduced. This expander is composed of a semipermeable silicon membrane that contains a hypertonic and highly saturated solution. Based on osmotic gradient and the difference in concentration between interstitial body fluid and the expander, the expander will expand due to influx of hypotonic tissue fluid. However, this technique has disadvantages, as the amount and the speed of the expansion was uncontrollable, and many other complications were mainly related to the fluid used. From this technical weakness, Wiese in 1993 developed an expansion system that still has the same self-inflating concept, but the model was based on hydrogel material, after which the hydrogel was suggested by Wichterle and Lim in 1960 as a biocompatible material and had been used successfully for many years in the clinical field. Osmotically driven self-inflating devices based on hydrogels are capable of generating sufficient force that is able to produce the required amount of tissue expansion. Those hydrogels became an interest in the field of reconstructive surgery. Hydrogel expanders were then modified by encapsulating them inside a silicone shell and subjected to compressive forces to

control the direction of the expansion to suit different anatomical locations and minimize the risk of device extrusion due to uncontrolled and multidirectional expansion (Swan et al., 2012)

Tissue expansion associated with molecular and biological changes in the tissue. Histological expanded tissues show numerous new blood vessels had been formed to provide more blood supply to the newly growing tissue and to accommodate the increasing surface area. On the other hand, countless studies have been reported that stress beyond physiological limits might lead to alteration in cell function and might also contribute to tumor formation (Wernig et al., 2003). Up regulation of inflammatory response occurs when cells are subjected to high-magnitude cyclic stretch, leading to leukocyte infiltration and cytokine production (Fang et al., 2013; Gawlak et al., 2016; Meliton et al., 2013). Inflammation is a part of the complex biological response of body tissue to harmful stimuli, such as pathogens, damaged cells, or irritants, and is a protective response consisting of changes in blood flow, increase in permeability of blood vessels, and the migration of fluids, proteins, and white blood cells (WBC) from the circulation to the site of tissue damage (Milenovic, 2011).

In spite of this, it is also crucial to note that inflammation and barrier-disruptive mechanisms are only brought on by high significant cycles of stretch and cannot be activated by means of physiologically associated cyclic stretch magnitude (Gawlak et al., 2016). Some witnessed that there was a reduction in macrophage infiltration and neutrophil migration when mechanical strain was performed (Berrueta et al., 2016; Corey et al., 2012), while others reported expression of pro-inflammatory cytokines in the absence of inflammation (Vlahakis et al., 1999). When tissues face expanded force tissue creep occurs (Chen et al., 2015). This deformity leads to changes in the intra and extracellular environments. Such changes provoke the release of biological signaling

mediators for tissue adaptation (Fleissner & Parekh, 2018), which leads to activation of cellular pathways to initiate the tissue response to the applied force (Elosegui et al., 2018). Several mediators are known to be involved during such a process, such as enzymes, hormones, neurotransmitters, and cytokines. All these mediators are a product of cellular signaling pathways and will act on one another. However, the same molecules within the same pathway are capable of producing a completely different result, depending on which receptor the ligand was bound and even pico level changes in the concentration will lead to shifts from an action to another.

It is still unclear how the cells respond to changes when mechanical strain is applied. Different types of stimulation work on different cells which leads to different results. For example, kidney cells and skin cells will respond differently even if both are subjected to the same steady expansion or mechanical force. However, experiments done on cultured cells instead of intact tissue are not able to clarify the precise results of tissue expansion (Razzak et al., 2016). Therefore, in this *in vivo* study we will focus on the molecular events of pro-inflammatory biomarkers in response to mechanical strain achieved by anisotropic self-inflating hydrogel tissue expander. Most previous studies were conducted clinically and focused on the methods and the techniques of using hydrogel expanders, and on the clinical success rate without explaining how our body deals with the devices from a molecular prospective. In addition, previous studies conducted experimentally to investigate the association between tissue when subjected to stretching force that leads to expansion and inflammatory responses were on cultured cells (Copland & Post, 2007)Hence, the purposes of this study are to investigate into details the inflammatory response following the tissue expansion process.

1.2 Hypothesis

Null hypothesis: There will be no difference in cellular pro-inflammatory cytokine production between the control and expanded tissues.

Alternative hypothesis

This study hypothesizes that the level of pro-inflammatory cytokines in expanded tissue will be different from non-expanded tissue.

1.3 Research Questions

i. Are there any histological differences between the expanded and control samples?
ii. Is there any inflammatory response in the expanded tissue?
iii. Do cells produce IL-1, IL-8, TNF-α, and IL-6 cytokines in the controlled and expanded skin tissue samples? If yes, where is the cellular location for these cytokines?
iv. Is the cytokine production between the expanded and non-expanded skin tissue samples identical?
v. Is there any association between inflammatory cells and cytokine production in expanded tissue?

1.4 Clinical Significance of the Study

Conventional tissue expansions in intra-oral and maxillofacial surgery are deserted compared to the benefits obtained from them in other parts of the body for many reasons. The anatomy and location of the mouth makes the use of conventional balloon-like tissue expanders inconvenient, making it difficult to reach and manipulate by the clinicians or patients themselves. Moreover, the most common procedure requiring intra-oral soft tissue expansion is when a significant amount of soft tissue is needed to

seal a dental implant (Atsuta et al., 2016). Atrophy of oral mucosa following tooth loss is very common among the patients, resulting in insufficient space to insert a large expander. The challenge is more difficult in cleft palate reconstruction due to its asymmetrical 3D slopes, resulting in variation in the thickness of the soft tissue covering between one area and another (Smahel et al., 2009). Additionally, proper speech is the main goal following repair of palatal function, yet it is hard to be achieved due to soft tissue tension secondary to a traditional flap (Nishikubo et al., 2009). Furthermore, patients with unilateral cleft lip and palate are commonly diagnosed with maxillary hypoplasia, thus Orthognathic-orthodontic treatment is compensatory. Adequate soft tissue is necessary to carry out complex orthodontic treatment to correct the malocclusion without gingival recession to facilitate tooth movement without its loss (Bhedasgoankar et al., 2011). Additionally, for successful intra-oral bone graft treatments, sufficient soft tissue covering with good vascular bed is compensatory to prevent the graft material from exposure and resorption (Wagener et al., 2019). The osmotically controlled tissue expanders have been promising to treat these conditions, where the success of balloon expanders has so far been limited.

1.5 Significance and Novelty of the Study

The current study investigates the inflammatory response following the use of anisotropic self-inflating hydrogel tissue expander *in vivo* for the first time. The use of anisotropic self-inflating tissue expanders will allow reconstructive surgeons to accurately and predictably control the time as well as the direction of the expansion. Thus, the timing and the rate of tissue expansion will significantly reduce the risk of soft tissue damage complications. Additionally, controlling the direction of expansion will make them ideal for use in delicate anatomical locations, particularly in children. As Professor Dr. Swan highlighted “There is always a clinical need for extra soft tissue in reconstructive plastic surgery, but until now there has been no reliable method of

attaining the optimal amount through hydrogel technology. This device will allow clinicians to treat more cases, at a lower cost, and hopefully with a better patient outcome. We also expect new procedures and clinical indications to arise as a result of this breakthrough technology."

1.6 Objectives

To investigate the histological differences between the expanded and control samples.

i. To investigate the inflammatory response of the expanded tissues.
ii. To detect the presence and cellular location of IL-1, IL-8, TNF-α, and IL-6 cytokines in the controlled and expanded skin tissue samples.
iii. To compare cytokine production between the expanded and non-expanded skin tissue samples
iv. To compare the inflammatory cell profile in association with cytokine production in expanded tissue.

1.7 Organization of the Thesis

After clarifying the background, objective, hypothesis, research questions and the clinical significance of the current study, this manuscript will give an overview of the available literature in the following chapter. In chapter two, three broad parts have been explored in the literature: historical background of tissue expansion until anisotropic hydrogel expansion, past intra-oral and clinical application of hydrogel tissue expansion, and general information regarding the biomarkers that were used in this study to detect inflammation. Additionally, the current study gives an explanation on the significance of conducting an *in vivo* study. Chapter three briefly elaborates and

explains the design of the study, sample selection, animal preparation, sample size calculation and sample preparation, methods, the process of data collection, and data analysis. Chapter four will elucidate the pivotal findings in histological and immunohistochemical analysis after the application of anisotropic hydrogel tissue expansion. Chapter five will discuss the results in relation with other findings from the literature. Lastly, the dissertation is concluded with future suggestions and recommendations for future research.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This section discusses the historical background of tissue expansion until the revolution of introducing hydrogel material as self-inflating expanders and the second generation of expanders (anisotropic hydrogel tissue expanders). This is followed by a review of literature on pre-clinical applications for, and clinical application of the device with a pointing out to their prominent features with some comments from the current study, these studies are organized based on the time period from 2009 till 2017, 2007 till 2015 respectively. A brief overview of our current understanding about the body response to when stress is applied. Following that, the methods of investigation of inflammation including histological investigation, brief information about the biomarkers and the common biomarkers that are used to detect inflammation, overview on cytokines proteins that are used as biomarkers in the current study, and the significant of inducting in-vivo study are discussed.

2.2 Historical Background

Neumann 1957 first introduced the concept of soft tissue expansion in reconstructive surgery to reconstruct traumatic ear defect (Nuemann, 1957). Confronted with the major disadvantages associated with the use of free graft either due to lack of good color, texture and even poor blood supply, he gradually increased the surface area of the skin and subcutaneous tissue by distention of an inflatable rubber balloon placed beneath the skin on the right side of the ear in a 52-year-old male to produce a donor tissue by expansion near the defective side to revise the problems associated with usage of free grafts. This technique renaissance after being used by Radovan and Argenta for

mastectomy in breast reconstruction (Radovan, 1982; Tepole et al., 2011). The ultimate general purpose of tissue expansion is to create ideal healthy tissue of normal texture, color, thickness with preservation of its sensibility in order to resurfacing of the defected site. Keeping a tissue under continuous tension leads to stretching, and change on cellular organization, which stimulate cellular division resulting in new tissue formation (Jamadi et al., 2017). Traditional expanders were improved by Radovan and Argenta in the 1970s (Radovan, 1982, 1984).The expander was a balloon–type with silicone envelopes which contained a self-sealed injectable tube. A normal saline solution was injected instead of air through a connecting tube to increase the size of the expander (Handschel et al., 2013), and since then this method becomes widely accepted and it is most appended technique to be used (Swan et al., 2011). However, higher complication rate was associated with this technique, the major complications were infection, expander exposure, wound dehiscence, and hematoma due to presence of blood under the large flap resulted in necrosis of the expanded and surrounding tissue area. Clinician training and patient awareness were factors which impacted and determine the success rate of the procedure. Thus this technique is uncontrollable and the protocol cannot be standardized (Fochtmann et al., 2013).

In view of this shortcoming, Austad and Rose in 1982, develop self-inflating tissue expanders based on the osmotically drive force of the semipermeable membrane made of silicon material containing saturated, hypertonic sodium chloride solution (Austad & Rose, 1982). Based on osmotic gradient and difference in concentration between interstitial body fluid and the expander, the expander will increase in size due to influx of hypotonic tissue fluid. The main aim was to materialize a device which expands the tissue and once it is placed, it requires no manipulation, no instrumentation, or relocation. The rate of the inflation was steady through the procedures and it is better in comparison to the conventional expanders which was still increasing in the treatment

period. However, the rate and the extent of the expansion were limited as the expander stop expanding once equilibrium is reached between the two environments. Thus, the desired expansion of tissue might not have been achieved. The clinicians have also noted a high risk of device rupture and soft tissue necrosis due to leakage of the solution. As a result, the main drawback of this expander was the amount and speed of the expansion that was uncontrollable. Confronted with this major disadvantage, the idea of self-inflated expanded was abandoned by clinicians, and tissue expansion in reconstructive surgery was only conducted using the conventional balloon type expanders filled with normal saline. Wiese in 1993 revive this concept again after he suggested using hydrogels which consist of modified copolymer as a self-inflated expanders instead of saturated saline solution (Wiese, 1993). Hydrogels have been used before and are known to be safe and biocompatible and they are often used by ophthalmologist as a material for contact lenses. Since then, self-inflation tissue expanders based on hydrogel had sparked the interest of plastic surgeons to expand tissue in a much more convenient way. Although the use of hydrogel expanders had successfully spread for clinical, the primary challenge which lies ahead falls on its design. In the first series of the expander model designed to expand identically in all directions, this isotropic property had markedly restricted its clinical application to only a specific area in the body. Moreover, the hydrogel has rate of swelling and increased in volume up to 1360% of its dry weight which is associated with the risk of device extrusion. To control the amount of the swelling, the hydrogels polymers were enveloped within a perforated silicon shell. A comparative study between osmotic tissue expanders with silicon shell and without silicon shell was performed in Germany using four pigs He found that the uncoated devices cause excessive soft tissue expansion that result in tissue ulceration. In contrast, silicon coated devices can produce controlled soft

tissue expansion with significant increase in the volume of expanded soft tissue with no evidence of inflammatory response to the implant (Anwander et al., 2007).

The second generation of hydrogel expanders that were covered with silicon shell were subjected to compression force only in one direction. Thus the swelling behavior will only be in the direction where the force was applied i.e. directional dependence (anisotropic). Hence, this modification will allow the use of the expander in different part of the body where the surface is flat, or in areas with different thickness (Swan et al., 2011).

Self-inflating tissue expanders based on hydrogel are synthesized by polymerization reaction of cross-linked hydrophilic polymers. The co-polymer that is produced is able to swell due to its supper absorbent property yet it does not dissolve due to its physical and chemical cross-links, tubular or crystalline regions. The swelling rate depends on the hydrophilic ability of the polymer, where the lesser the cross linking density and the more free space in the networks, the swelling rate will be increased (Jamadi et al., 2017). Many types of hydrogel expander are available depending on the types of the polymers material incorporated In this study a methyl-methacrylate and N-vinylpyrrolidone copolymer (AMM-NVP) based anisotropic self-inflating hydrogel is used, in ratio of 10:90 respectively, the additional type of polymerization reaction is initiated by azobisisobutyronitrile (0.2 wt.%), and the formed gel is processed from isotropic to anisotropic as the following; The gel is subjected to high temperature at 161c˚ in thermostatically controlled hydraulic press glass chamber for about 60 mins, then allowed to cool down for another 60mins under the compressor at room temperature (Swan et al., 2011).

Anisotropic self-expander is more precise and targeted for the purpose of tissue expansion. The final size is predictable and can be controlled by customizing its initial proportions, and the speed of expansion can be modified by the composition of the polymer (Garner et al., 2017). (Table 2.1), and (Figure 2.2).

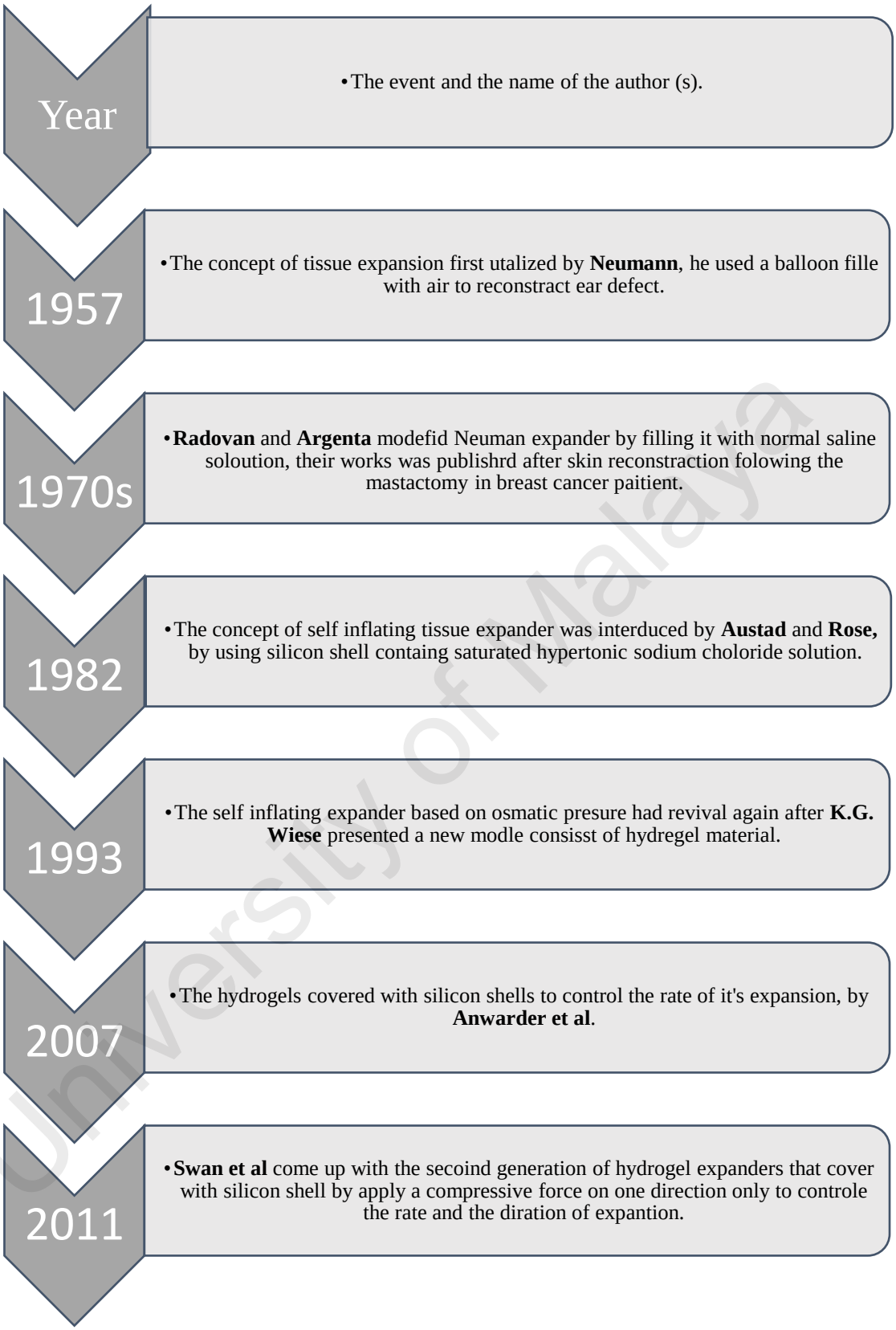
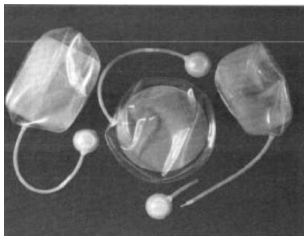
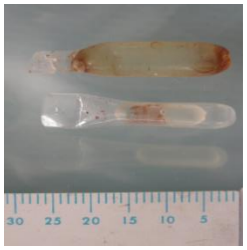
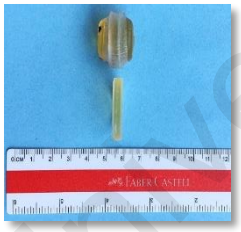


Figure 2.1: Historical backgrounds of tissue expanders until the investment of anisotropic hydrogel tissue expander

Table 2.1: Expander category, types, and comments related to the type of the expander

Expander category	Type	Comments
Silicon balloon 	Inflated with Air Or Normal saline	. Silicon balloon. . Large size. . Increase in size by regular inflation. . Long treatment time. . Associated with high complication. . Risk of external infections.
Hydrogel 	Self- inflated	. No silicon envelope. . Small size. . Increase in size by osmotic pressure. . Rapid, uncontrollable swollen rate. . Devise extrusion. . Depends on the site, might be associated with high complication
Hydrogel 	Self- inflated	. With perforated silicon envelope. . Small size. . Increase in size by osmotic pressure. . Rapid, controlled swollen rate. . Expand in all directions. . Devise extrusion.
Hydrogel 	Self- inflated	. With perforated silicon envelope. . Small size. . Increase in size by osmotic pressure. . Rapid, controlled swollen rate. . Expand in one direction. . Less Device extrusion.

2.3 Pre-clinical Experiments on Animals

Abrahamsson and co researchers from the department of Maxillofacial Surgery, evaluate the intra-oral soft tissue characteristics after expansion using the self – inflatable expander (silicon envelope with a flat end, 2.5mm wide, 7.5mm long, 3mm thick) in eight adult Swedish loop rabbits (one female and seven male). The isotropic self-inflating expander placed under the periosteum of the mandible through an extra

oral approach via incision was made bilaterally (roughly 15mm) at the lower border of the mandible to prevent scar formation. Followed by blunt dissection to the periosteum, the periosteum was cut in the direction of the lower border of the mandible. The expander was placed away from the suture line in a pocket created under the periosteum. The expander was left to self-inflate for two weeks, after which the animals were killed and specimens collected. Clinically there was no dehiscence or infections. In terms of histological findings, all sections were stained by Goldner stain and scanned by light microscope. The samples show thickening of the epidermis and thinning of the dermis (Abrahamsson et al., 2009). However, the finding on this study was not in agreement with other studies, where the epidermis thinned and the dermis thickened. This could be due to the fact that the tissue was oral mucosa which might be the reason way the tissues respond differentially to the force applied by the tissue expander.

One year later the same team made another study on 13 adult female Swedish loop rabbits. They wanted to use microporous titanium membranes to facilitate more bone regeneration in comparison with microporous meshes. Nevertheless, adequate soft tissue coverage was required for the grafted bone and the titanium to avoid exposure which may result in loss of the bone graft. They used osmotic tissue expander with a silicon shell (2.5 in wide, 7.5mm long and 3mm thick) to get a predictable localized donor tissue. In the histological analysis of decalcified bone stained with Goldner staining investigated using a light microscope with an integrated camera, there was no inflammatory cell response except in two rabbits with exposure of the meshes where moderate inflammation could be observed. However, newly formed bone and cortical bone was seen with addition of bone island, and new blood vessel was developed in direct contact with the mesh used (Abrahamsson et al., 2010). From the previous studies we can assume that the force generated triggers cellular growth and formation of new cells from the macromolecular point of view.

Von See and co-workers in 2010 found that a pre-augmentation soft tissue expansion with hydrogel expanders lead to higher functional microvessel density in tissue above the augmentation material, thus more rapid formation of osseointegration and no inflammation response was found in their study of sixteen isogeneic Lewis rat. They randomized them in two groups, group one animal (n=8) and group two animals (n=8). Additional eight animals served as donors of isogenic bone grafts. The bone grafts were harvested and implanted into group 1 (without tissue expansion) and in group 2 (after tissue expansion). Hydrogel expanders with a silicone shell were inserted subperiosteally at the site to be augmented at the Calvaria for 21 days. They used intra-vital microscopy to monitor microcirculation *in vivo* for 19 days after implantation. Decalcified bone specimens from both groups were evaluated histologically by light microscope in hematoxylin and eosin stain. None of the animals had microscopic inflammation in contrast to the group without previous tissue expansion. A thin zone of granulation tissue was found between the calvaria and the augmentation material. Partial resorption of the calvaria bone led to remodeling of the cortical bone and exposure of the underlying bone marrow. The increase in blood perfusion was significantly higher in the group with tissue expansion prior to augmentation. Thus, good vasculature is necessary to prevent bone augmentation from resorption (von See et al., 2010). This literature concluded that the hydrogel was a biocompatible material and confirms the finding that was reported by Wiese 1993.

Similarly, Henri and his co-workers implanted Osmed self-inflating soft tissue expanders in 25-goat maxilla in 2011. The osmed expanders with silicon envelope were implanted in 25 goats using a tunnel and flap approach. The animals were sacrificed after 1h (control) and 40 days (treated). Eight tattoos where placed on the frontal skin before the surgery and two after .The tattoos were still visible after 40 days, although some of them faded slightly as a result of tissue stretching. The soft tissue did

not expand symmetrically leading to an asymmetric pattern of the tattoos. Histologic evaluation of the palatal soft tissue stained by (Mc Neal solution and Fuchsin solution) showed no inflammation at any time in animals under the light microscope, and all of the wounds healed completely without complications. The tissue expanders in all goats had been in situ during the 40 days and clear palpable expanded palatal mucosa is observed, not clinically different from the unexpanded tissue(Uijlenbroek et al., 2011). They concluded that due to the shape and the direction of the expander, the device requires modification as this might restrict it's application on some parts of the oral cavity, thus they further suggested that for future studies, evaluation on the possibility of using osmed hydrogel expander with the silicon shells on a flat intra oral surface can be conducted.

In 2011, Wysocki et al performed a comparative study to investigate the biomechanical properties (maximum tangential, maximum tangential modulus and tensile strength) of expanded mucoperiosteal palatal tissue after the regimen of rapid expansion for 48 hours with the use of osmotic expander and correlated with histological finding. New Zealand white rabbits 20-40 weeks old were used in this study. This animal models were chosen due to its palate size, about 12-14mm wide, matching the width of palatal shelves of cleft palate in a child to create comparable conditions of tissue expansion and utilization of the same size expanders. The groups were divided to four: group C (non-operated controlled group), group C1 (sham control group where the mucoperiosteal tissue was elevated without expander implantation via blunt sub-periosteal dissection), group A-24 (24 hours expanded), and group A-48 (48 hours expanded). Histological examination of the soft tissue section that was stained (Hematoxylin and eosin) under the light microscope showed inflammation and tissue necrosis only in the healing phase of 48 hours in the expanded group. This is a result of mechanical tissue damage due to excessive strain and stress, which led to deterioration

of blood supply. In addition, increase in collagen density was also observed (Wysocki et al., 2011).

Manssor et al., 2016 evaluated the effect of tissue expander on skin tissue samples on the adult male Dorper sheep (2 years old). Following the expansion, Manssor et al, evaluated the characteristics of the skin collagen fibrils the skin samples visualized by atomic force microscope. Significant difference was detected when the results were compared to the control group as the fibrils from the expanded tissue consisted of loosely packed collagen fibrils with the width of the fibrils significantly narrower compared to those from the normal skin (Manssor et al., 2016).

Another study was made in 2016 to evaluate the impact of anisotropic self-inflating tissue expander on sheep scalps. The expanders were implanted subcutaneously on sheep scalps for 21 days before the animals were killed and the skin samples harvested and evaluated histologically. When compared to the control group, it was reported that there was no statistical difference in the dermal thickness of the sheep group treated with tissue expander (Aziz et al., 2017).

In 2017, a study was carried out on Beagle Dogs to examine mucosal vascularity following the insertion of the hydrogel tissue expanders. The expander was placed submucosally under the buccal surface of the alveolar ridge at the premolars region of both the mandible and maxilla, four months following extraction of all the upper and lower premolars teeth. After 42 days, the expander was removed, tissue biopsies were taken to assess the blood profusion, presence of acute or chronic inflammation and the types of inflammatory cell involved were determined. Histological results showed no inflammation with an increase in the vascular density suggesting that neovascularation had taken place (Barwinska et al., 2017).

A new formula of hydrogel tissue expanded created by Garner and his coworkers, by increasing both of the hydrophobic polyester content and crosslinking density, the swelling rate and volume as well as the mechanical strength was increased. The expanded skin samples from 18 Sprague Dawley rats were compared with other controlled expanded samples by osmed expander (same used in this study). The study concluded that the expanded flaps by osmed material had higher vascularization at 6 weeks post expanded compared to the expanded flaps by the new hydrogel formula (Garner et al., 2017).

2.4 Clinical Applications

Kobus has performed the first clinical trial for hydrogel tissue expansion intraorally. He conducted a surgical procedure in 19 children aged from two to three years old in British to repair their cleft palate. Non-enveloped, osmotic, rapid hydrogel expanders were left in patients for about 48 hours. Clinical observations showed that there were tissue necrosis, and cyanosed tissue. However, the tissue regained their color almost immediately after the removal of the expander. Eight of patients' clefts were closed and two developed mini-fistulas on the border of the soft and hard palate. Due to rapid expansion, the patients were under continuous investigation and measurements of the swelling rate based on recommendation of the manufacturer time were taken to prevent extrusion of the device. The expansion was rapid and aggressive, even beyond the mucoperiosteal coverage of the hard palate towards nasal cavity. As a result, the risk of nasal perforation was very high. Furthermore, narrow and compact areas such as alveolar process and palatal foramen were not expanded. Apart from this surgical procedure and post-operative course, there was no complication observed in early follow-up examination after 4-6 months (Kobus, 2007). From this study, we found that the author suggested that there is a need to modify the technique to control the expansion direction, and he found that using a rapid expansion is associated with higher

risk of complication. On the other hand, comparing with the time that the conventional expansion reach from 3 to 6 months, he finds that the hydrogen expanders have more advantages, and for him he need more sample size in order to give conclusion".

In 2011, Kaner and Friedmann had conducted a study to reduce the post rehabilitations complication by using osmatic soft tissue expander (STE). Their methods were to use the expander before vertical bone augmentation for the first time in patients seeking implant treatment in Berlin. These patients have resorbed edentulous or partially edentulous ridges. They found that the combination of STE and subsequent vertical augmentation provide high gain of well-structured bone for further successful implant therapy and minimal complications. Histological biopsies taken from the expanded soft tissue surrounding the expander stained with Hematoxylin\eosin, investigated with a light microscope showed a fiber-rich dens connective tissue without the presence of inflammatory cell, and new periosteum is formed below the expander and other cases shows subperiosteal resorption, those, as the results were not expected by the researchers (Kaner & Friedmann, 2011). However, no report about what is the possible cause of completely two different evidences. This could bring us to the complex cascade phenomenon of the different signaling pathways that might be activated by the mechanotransduction force even if the same force was applied to the same cells.

Similar study was conducted on expanded mucoperiostum palatal tissue using osmotic tissue expander with silicon shell cover. However, this was focused on the treatment of difficult anterior palatal fistulas. It was carried out in 2011 by Jenq et al. on seven patients from Oregon Health and Science University (OHSU), Portland, Oregon. All patients underwent a two-stage procedure under general endotracheal anesthesia. The first stage was placement of the expanders, the second stage was 1 week later with

removal of the expanders after 7 days. Five patients had complete closure of the fistula, two patients demonstrated slit like fistulas that asymptomatic. No complications were observed, there were no instance of flap loss, inflammation or necrosis (Jenq et al., 2011). The authors from this mentioned literatures alerted an important fact, with their techniques of using raped expansion, there was a higher risk of nasal perforation within the thin palate bone, such as perforation due to the all directional of expansion. This opinion is in agreement with Kobus 2007 in previous literature mentioned above.

Between November 2004 and September 2009, total of 30 children and adolescents underwent reconstructive surgery for burn scars, congenital nevi, alopecia and food deformities. The objective of Bottcher eat all is to gain sufficient amount of additional skin for the intended coverage of the defect, thus self-inflating hydrogel tissue expander was inserted in difference anatomical location including scalp, back, face, neck, foot, lower leg, shoulder, left knee, right and left forearm. The nature of the expander minimized the risk of infection, lowering the cost, and the numerous painful distressing filling sessions were obviated particularly in children (Bottcher et al., 2011).

Abrahamsson and his team in 2012 had used the isotropic hydrogel tissue expander prior to bone augmentation for 10 patients who require dental implants. Due to soft tissue gain, it was possible to add bone grafts in vertical and lateral dimension. There was no resorption on the augmentation bone which indicates that the tissue generated provide good vasculature and support to the augmented bone graft. However, 20% of cases showed perforation of the expander (Abrahamsson et al., 2012). Similarly, Mertens in 2015 evaluated the success rate of using the hydrogel tissue expansion before to augmentation with bone block grafting. However, to minimize the rate of perforation, device extrusion and necrosis, the expanders were left in the patients for about 90 days. Thus, long-term procedure was not convenient for the surgeon and

nether for the patients, she suggested to modify the technique and the device (Mertens et al., 2015).

The characteristics of expanded skin and the quality of their cellular, vascular and cytoskeletal had become the main concerns among surgeons. Multicellular organisms are totally dependent on active networks of extracellular signal molecules such as growth factors, hormones, neurotransmitters, and cytokines which are designed to orchestrate cellular communication for normal tissue hemostasis regeneration and repair. Controlled mechanical overstretched skin yield distribution in cell signaling, yet it also affects the cellular behavior (Kippenberger et al., 2018; Sethi et al., 2017). When the tissue is stretched, mechanotransduction pathways are activated (Abuammah et al., 2018; Elosegui et al., 2018; Fleissner & Parekh, 2018; Hsu et al., 2018; Sethi et al., 2017; Trubelja & Bao, 2018). This will result in cellular growth and new vascular formation (Trubelja & Bao, 2018). However, some studies showed that cyclic deformation resulting from external stretching does stimulate production of proinflammatory cytokines even in the absence of cellular damage or death. The mechanical force resulted from implantation of soft tissue expander lead to peri expander fibrous capsule formation as result of foreign body response mediated by immune cells and cytokines (Sheng et al., 2014).

Generally, when the cells are subjected to stressing force, changes occur on their surfaces due to internal and external environmental osmotic pressure (Mathieu & Manneville, 2018). Injury and cellular response will vary from reversible conditions such as mild cellular damage and edema or irreversible conditions where cell death occurs if the cell was unable to repair itself. For the tissue to maintain its function, the damaged cell will be repaired, regenerated or replaced by another cell or connective tissue (Lee et al., 2018; Wang et al., 2017). Chemical mediators are needed for this

process. The released chemicals will also trigger inflammation as it plays a central role in the healing process and body defense. Depending on which pathway where the cells were activated, different chemical molecules will be released, and thus, different tissue response will be obtained. The released signaling molecules will in turn lead to different physiological/pathological response depending on which receptor they will bind (Figure 2.2).

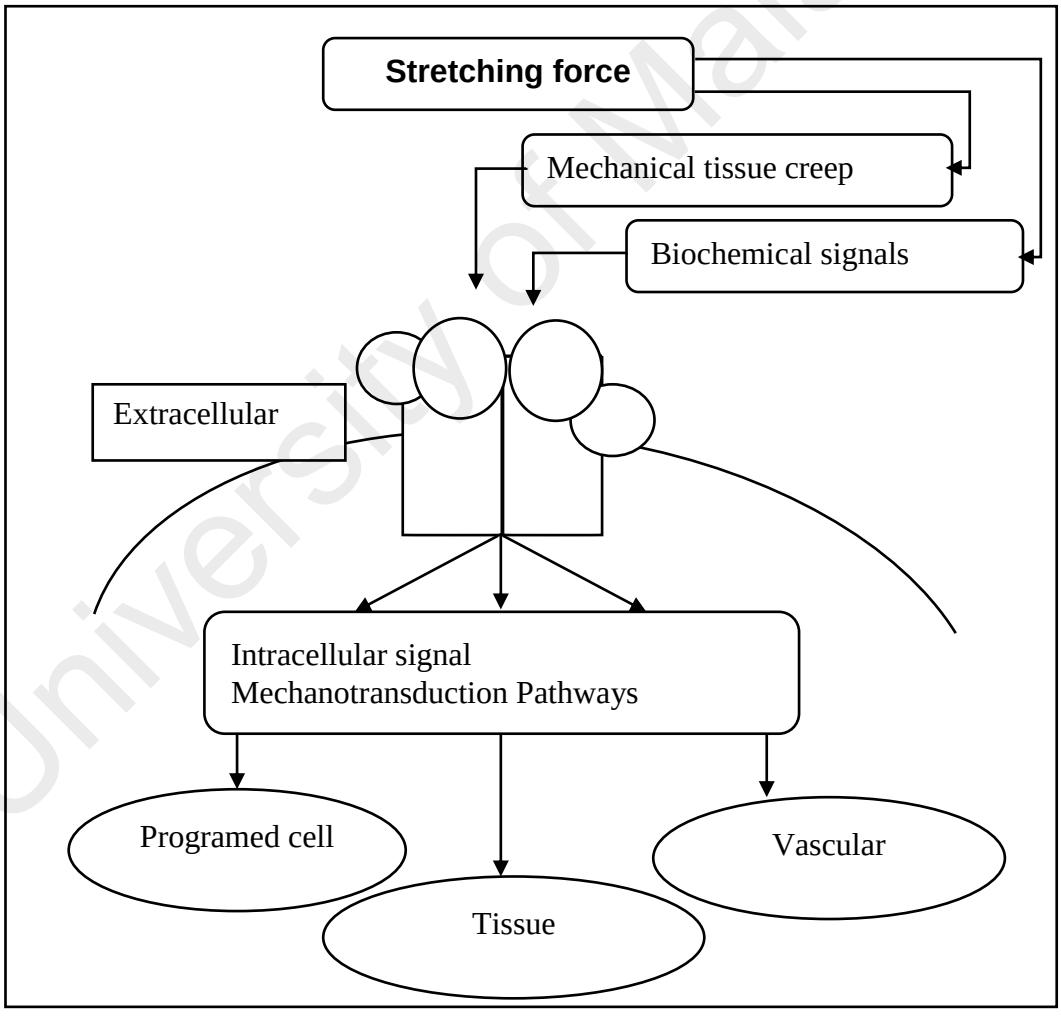


Figure 2.2: Schematic representation of different cellular responses via signal transduction pathways due to the effect of tissue expansion

When the ligand binds with the cell receptor, activation of intracellular signal mechanotransduction pathway starts its cascade phenomena. How and what is the mechanism involved, is still not fully clear. Whether if those mediators arise from the AKT signaling pathways that are mainly inhibited by apoptosis or these biological signals initiate TGF-Beta pathway to facilitate tissue homeostasis and cellular growth. it is still unclear to their secretion will trigger TNF – superfamily pathway to direct a programed cell death. These questions needed to be answered in direct relation with tissue expansion in general yet anisotropic hydrogel tissue expanders specifically. Tremendous studies were made and they were focused on the clinical application of the hydrogel tissue expander and the microscopic investigation on the cellular changes, but there are no reports which address the mechanism behind this response, neither the growth nor other cellular changes. In this study, we will investigate the direct relation between the novel anisotropic hydrogel tissue expander and the inflammatory process. We believes that our results will give a useful information for better understanding on how dose our tissue physiologically/pathologically response to the expansion from macromolecular point view.

2.5 Method to Investigate Inflammation

Skin inflammation can be conventionally diagnosed indirectly and predominantly clinically by assessing the inflammatory symptoms. However, more direct methods can be involved.

2.5.1 Histological Analysis

In diagnostic routine of skin pathology, H&E staining is the most common used stain in Histology Laboratories (Otali et al., 2016). It is a fundamental stain for a general appraisal of the skin and tissue morphology (Al-Refu, 2011). H&E produce a varying staining intensity of cell cores ranging from blue to dark purple, while the cytoplasm

and the connective tissue fibers appear pinkish-red or orange color to display a broad range of tissue features(Caiwen Li & Chen, 2017). Recently, there have been several noticeable advances in developing a fluorescence based methods to visualize H&E stain by electrophoresing due to increase in interest and demand to the stain in dermatology (Verma, Tripathi, & Gangenahalli, 2016). The ability of the stain to recognize various types of tissue and differentiate the morphological different of the living tissue makes it one of the powerful diagnostic tool then and now. Among dermatologists, H&E is considered as a principle stain for studying normal and abnormal skin, diagnosis of many dermatological diseases, post evaluation in treatment planning (Zulfakar et al., 2011). Immunohistochemistry on the other hand, allows direct visualization of the targeting markers on the tissue. In addition, this study will focus on these two methods.

2.5.2 Inflammatory Biomarkers

Biological markers (Biomarkers) are indicators used to measure sub-organismal response to any changes in their biological systems (Bethesda, 2001). Biomarker could be helpful to estimate and assess the biological, pathological, and genetic process of the living organelles (Allen, 2017). Moreover, biomarkers are also used as diagnostic tools for certain diseases and help in classification and differentiation of one disease from the other. These indicators are tested using blood, serum, plasma and tissue (Au, 2004). Recently, the science of biomarkers has make progress noticeably. Indeed, implanted and injected biomaterial prompt a wide range of adverse effects that are inflammatory in nature and commonly associated with white blood cells accumulation as tissue response. Therefore, to understand this response, using the biomarkers as investigators for inflammation in the use of anisotropic hydrogel tissue expansion is a high priority. The most widely investigated and promising biomarkers for inflammation are cytokines, as cytokines produced many cells to initiate and/or in response to inflammation. Besides cytokines, other indicators are also used such as C-reactive protein (CRP), monocyte

chemoattractant protein-1 (MCP-1), matrix metalloproteinase, growth factors and reactive oxygen.

2.5.3 Cytokine Proteins

Cytokines are low-molecular-weight proteins (Ferreira et al,. 2018) (glycoproteins with molecular masses of 15,000–30,000 Dalton) that regulate the immune system and inflammation by a complex and highly coordinated inducted cascade process. They bind to specific receptors on target cell membranes to trigger signal transduction pathways that ultimately result in altered genetic expression in target cells. An important feature of cytokine biology is that cytokines and their receptors have very high affinity for each other. A minor picomolar changes in cytokine concentration can also mediate significant physiological effects (Feghali. & Wright., 1997). Cytokines are produced by immune and non-immune cells in response to inflammation and cellular damages in order to attract white blood cells to the affected sites. Migration of immune cells increased and plasma level of cytokines are elevated (Wang et al., 2017). On the other hand, cellular growth and development are also under direct control of cytokines. Cytokines could either be initiatory (pro-inflammatory) or inhibitory (anti-inflammatory) depending on their action towards inflammation. There are many pro-inflammatory cytokines proteins that are involve in inflammation but in this study we will focus only on IL-1, IL-6, IL-8 and TNF-α.

2.5.4 Interleukin-1

Interleukin-1 is a multifunctional cytokine, targeting mostly all types of tissue cells. The presence of IL-1 is always correlated with TNF-α (Ralf et al., 1990; Feghali. & Wright., 1997). Interleukin (IL) - 1 was originally described in the 1940s as a fever-inducing molecule secreted by activated leukocytes. Previously it was known as “pyrexin” or “endogenous pyrogen”. In addition, it was first cloned in the 1980s, as an

impact role on inflammatory regulation (Gabay et al., 2010). Activated macrophages are considered as the major source of IL-1.However, many other types of cell such as epithelial cells and endothelial cells can produce IL-1. There are more than 50 IL-1 superfamily members (Dinarello, 2011) and they are classified base on their structure and biological function (Jenei et al., 2018). The common active forms of IL-1 are IL-1α and IL-1β (Mantovani et al., 2018; Akdis et al., 2016), but their biological activities are identical. Despite the similarity in their action, these two are mainly different in their location (Garlanda et al., 2013), IL-1β is circulatory in form and found in the blood, whereas IL-1α is generally associated with the plasma membrane of the secreted cell and acts locally (Sims & Smith, 2010). IL-1 binds to special receptors, which are estimate to be around 10 receptors. They are found mostly in all living cells as 95% of body organs use innate immunity as a defense mechanism (Dinarello, 2018). Under normal conditions, the secretion of IL-1 requires induction at both the transcription and translation levels. Following the release of active cytokines from the cell, the action is blocked by two physiological mechanisms of IL- 1- binding protein, such as IL-1 receptor antagonist (IL-1Ra), IL-1 receptor type II (IL-1RII) (Gabay et al., 2010). On endothelial cells, the function of IL-1 is to promote the expression of surface molecules that leads to adhesion of white blood cells to the walls. Moreover, IL-1 enhance the macrophages and smooth muscle cells to secrete chemokine to attract white blood cells, Hence, the increased in the body temperature can stimulate the hepatic to synthesis acute face proteins (Schindler. et al., 1990).

2.5.5 Interleukin-8

Interleukin-8 or (CXCL1) belongs to the CXC family of Chemokines, Chemokines are small macromolecules, approximately (8–10 kilo Daltons), soluble, basic, heparin-binding protein. Expression of IL-8 is tightly regulated, therefore, their level is normally undetectable or low in the tissue. The CXC is characterized by the presence of

amino acid terminal which has separated the two cysteine residues contain an amino acid sequence Glu-Leu-Arg (ELR) preceding the first conserved cysteine amino acid residue in the primary structure of these proteins (Hawwa et al., 2011; Pedersen et al., 2007). IL-8 has high chemotactic ability (Brown et al., 2010; Oppenheim et al., 1991), and powerful chemotactic factor on neutrophil (Ray & Kolls, 2017). It is produced by the cells to trigger neutrophil adhesion and activation at the inflammatory site (Asa'ad et al., 2018; Bhedasgoankar et al., 2011). It has been found that activated White blood cells such as monocytes and macrophages appear to be the banded cellular sources of IL-8 (Bester & Pretorius, 2016). However, several non-immune cells, such as endothelial cells when exposed to pro-inflammatory cytokines, and keratinocytes epithelial cells can also produce this chemokine (Giribabu et al., 2017). Additionally, IL-8 is considered as an angiogenic growth factor that influences smooth muscle cells proliferation and migration (Hou et al., 2014).

2.5.6 Tumor Necrosis Factor-α

Tumor necrosis factor was discovered in 1975 because of its ability to reduce tumor cells growth in mice known as "cachecitin/ cachexi" (Mukai et al., 2018). Later in 1984, the same molecule was purified and cloned by Kruglov and his team (Haider & Knofler, 2009; Kruglov et al., 2008). It plays a key role in innate immunity responses to infection. Moreover, its function is critical in many biological conditions, maintaining body hemostasis (Kalliolias & Ivashkiv, 2016), and it also has a central role in the neurological system (Sedger & McDermott, 2014). The 17-kDa soluble TNF-α is the mature form which mediates biologic effects. TNF- α is first synthesized as 26-KDa trans-membrane molecules and can be cleaved by the TNF-α converting enzyme, releasing the active soluble 17-KDa form. The 26-kDa trans-membrane pro-TNF-α however, is also considered active and has been shown to mediate the cytotoxic effect of TNF-α through cell-to-cell contact (Hu et al., 2014). Different cell types following

different stimulants secrete TNF-α (Bercier & Grenier, 2019), though LPS was found to be the most effective inducer. Additionally, even though zymosan and opsonised RBC induces TNF-α secretion by macrophages, they are 10 times less effective than LPS. On other hand, not all have the same efficiency, as TNF-α release or mRNA accumulation depends on both the degree of macrophage activation and the nature of induction. Lymphocytes are also an important source of TNF-α after stimulation of T-lymphocytes by anti-CD3 in association with IL-2 or mitogens. NK cells represent a widely disseminated and large potential source of TNF-α. Several studies concluded that mast cells are unique as a source of TNF-α, as they contain TNF-α in their granules. After stimulation by immunoglobulin IgE the cells degranulated and secrete it (Moon, Befus, & Kulka, 2014). Polymorphonuclear leukocytes (PMN), UV light and LPS exposed keratinocytes show accumulated TNF-α mRNA and release TNF-α. The non-neuronal cells, i.e., neuroglia of the central nervous system (astrocytes and microglial cells) release TNF-α as well as IL-1 and IL-6 after being induced by LPS and some neurotropic viruses (Vassalli, 1992). All the cell types of the body have two different types of receptors, type I (TNFR1) and type II (TNFR2) that mainly interact with TNF-α to initiate its biological functions (Sedger & McDermott, 2014). When produced in small amounts, TNF-α acts locally as a paracrine and autocrine to regulate white blood cells and smooth muscle cells of vascular walls. It has a principal role to activate the vascular endothelial cells to secrete adhesion molecules and to facilitate neutrophil, monocyte and lymphocyte migration. Moreover, it has a potent effect on chemokine secretion by macrophages and leukocyte activation (Haider & Knofler, 2009). In larger concentrations however, TNF-α enters the circulation and leads to wide systemic effects, increasing synthesis of prostaglandins in the hypothalamus, stimulating macrophages and vascular smooth muscle cells to secrete IL- 1 and IL-6, and

stimulating the liver to synthesis acute phase proteins, resulting in an increase in body temperature (Haider & Knofler, 2009).

2.5.7 Interleukin-6

IL-6 is also known as B cell activated factor due to its ability to stimulate B cells to produce immunoglobulin. It is one of the most common cytokines that are involved in all different types of inflammation. It has both pro- and anti-inflammatory action on the inflammatory process. It's made up of 212 amino acids where 28 of those amino acids are single peptides. Although mainly immunological cells like T cells, B cells, and monocytes produce it, several non-immune cells are considered to produce a large amount of IL-6 during the inflammatory process such as fibroblasts, keratinocytes, endothelial cells, and bone marrow cells. Adipose tissue in obesity produces IL-6 when the fat cells expand. Muscular tissue secretes IL-6 during the healing process after muscle fatigue. The normal serum level of IL-6 in healthy individuals is <3-4 pg/ml. Together with other cytokines, IL-6 has an important role in acute inflammatory response. It stimulates the liver to produce acute phase proteins like c-reactive protein (CRP) which in turn has a significant role in increasing body temperature and inducing fever. On the other hand, it is crucial to understand that IL-6 also plays a central role in cellular regeneration, proliferation, and maturation. Hemostasis, bone marrow metabolism and the vascular system are directly under control of IL-6. Several studies showed that IL-6 plays a role in endothelial cell proliferation and new blood vessel formation. It has been pointed out that this cytokine has a direct relation with angiogenesis, even in the absence of inflammation. IL 6 is also known as B cell activated factor due to its ability to stimulate B cells to secrete IgG. It is important to know that there are several members of the IL-6 family, each of them acting in different cellular signaling pathways, which include; IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary inhibitory factor (CNTF), cardiotropin-1 (CT-1),

cardiotrophin-like related cytokine, stimulating neurotrophin-1/B-cell stimulating factor 3 (NNT-1), neuropoietin (NPN), IL-27, and IL-31. With the exception of IL-31, all IL-6 type cytokines share the membrane glycoprotein gp130 as a common receptor (Scheller et al., 2011).

2.6 The Importance of *In Vivo* Study of Anisotropic Self-Expander Hydrogel Tissue Expander

Scientists use a variety of methods and tools to perform experiments on skin cells to study the development, differentiation, function and cellular response (Xua et al., 2018; Zhou et al., 2018). These experiments could either be conducted inside the living organism (in-vivo) or takes place on part of the tissue cells after they had been cultured (in-vitro). The later is more commonly used in scientific research. However, those tests are unable to accurately predict results similar to living organisms, as the condition is different. In contrast, in-vivo studies enable the researcher able to observe the overall effects of living subject in its natural environment (Coulomb & Dubertret, 2002). Therefore, our in-vivo study will be able to give more persuasive results that can actually represent the actual body response. Hence the final data will be free from doubts.

CHAPTER 3

METHODOLOGY

3.1 Ethical Approval

This study was ethically approved by the Faculty of Medicine Institutional, Animal Care and Use Committee (FOM IACUC), University of Malaya. Ethics number 2015-161006/DENT/R/ZR.

3.2 Study Design

This was an experimental study designed to examine the effect of anisotropic hydrogel tissue expansion on inflammatory mediators and the possibility of cytokines to be induced as the body responds to the expander. This study utilized rat skin as a model.

3.3 Sample Selection

Skin samples were taken from Sprague Dawley rats (300g ± 50g). Rats were chosen for many reasons. First, they share 99% genetic similarities with humans. This will ensure that the macromolecular data will provide useful information for future clinical use. Additionally, they were large enough to make them easy to handle for surgical procedures. The age of the rats was 8 weeks, therefore eliminating any possible compounding factors resulting from growth. Moreover, biochemical materials, reagents, and biological markers are commonly available and easy accessible for those species.

3.4 Animal Preparation

Seven, 8 week old female rats were used in this study. 3 rats were used as controls and 4 were used for *in vivo* expansion. The hair of the scalp region was removed and the scalp was permanently tattooed by using indelible ink and a tattooing machine to allow

accurate measurement of the degree of swelling throughout the expansion process. Local anesthetic using 3-5 ml ketamine per total body weight (kg) (Vetoquinol, UK) and 1 ml of Diazepam (Diapine, Atlantic Labs, Thailand) by intra peritoneal administration was administered to achieve the anesthesia and to improve post-operative analgesia and minimize bleeding. A small skin incision on the frontal region was made and a subcutaneous pocked was created by blunt dissection. A sterilized tissue expander (Oxtex, UK) was implanted in the pocket at the midline of the scalp for the expanded groups. Each expander was of known dry mass and was a cylindrical device up to 30 mm in diameter and 10 mm in thickness. The surgical wound was sutured (3-0, Silk, reverse cutting needle, Unik, Taiwan) and dressed. While for the control groups, the surgical site was sutured without placing of the expander. Post-operative monitoring and appropriate post-care including maintenance of temperature (20-26C°), other environmental conditions and analgesia was conducted according to Institutional Animal Care and Use Committee (IACUC), UM.

Following recovery from the surgical procedures, the rats were returned to the cages when they have gained consciousness. Regular visual inspection of the surgical site was performed for detection of post-operative complications. The rate of device swelling was monitored by regular visual inspection and simple measurement using Vernier caliper (Mitutoyo, Japan). Animals were monitored for a period of four weeks according to the guidelines for animal care and use (IACUC, UM, Appendix D), after which they were sacrificed.

3.5 Sample Size Calculation

Sample size calculation was based on “resource equation methods” (Charan & Kantharia, 2013). According to this method, (E) which is the degree of freedom is measured by the following formula: E = (total number of animals × repetition for each

group) − total number of groups. Based on Charan and Kantharia, this method is applied on animal experiments where the rejection of the null hypothesis is not the main focus, and the researcher only wants to see the difference between the groups. Thus, the sample size which keeps E between 10 and 20 is considered adequate. However, E should not be more then 30. In this study three control, three expanded animals, and one for negative control test were use. The repletion of each animal was four times. Hence (E) was 25, which is considered adequate.

Based on (Resource equation methods) (refer to equation 3.1):

$$E = (total\ \text{"n"}\ of\ animals \times repetition\ for\ each\ animal) - total\ n\ of\ groups) \quad (3.1)$$

$$(7 \times 4) - 3 = 25$$

3.6 Sample Preparation

Samples from expanded and controlled rats were taken immediately after they were sacrificed. Expanded skin sample measuring 2cm^2 were excised and fixed in 10% formalin solution overnight at room temperature. Control samples were also collected from rats of the control group which were not subjected to expansion. Then embedded in paraffin wax. 5µm sections were then cut by microtome at room temperature and mounted on polarized saline coating glass slides. For each paraffin wax block, one slide was assigned for histology and four for immunohistochemistry. All the procedures were performed at the Oral Pathology Laboratory, Faculty of Dentistry, University Malaya.

3.7 Methods

3.7.1 Antibodies Preparation

Upon receipt, the antibodies (Table 3.1 and 3.2) were centrifuged at 10,000 RPM for 20 seconds to ensure any solution that may be trapped in the threads of the vials were

collected at the bottom. The antibodies were then immediately aliquoted (50 µl) in sterile tubes (Eppendorf) and stored at -80°C.

Table 3.1: Primary Antibodies

Name	Description	Concentration	Producer	Suitable for
Ab124962 (IL-1α)	Rabbit monoclonal (epr6483)	100µL In liquid form	Abcam	+ (ELISA) 1/100(IHC-P)
Ab208113 (IL-6 receptor)	Rabbit polyclonal	50µg Liquid form	Abcam	1/1000(ELISA) 1/50 (IHC-P)
Ab7747 (IL-8)	Rabbit polyclonal	500µL Liquid form	Abcam	+ (ELISA) 1/100 (IHC-P)
Ab6671 (TNFα)	Rabbit polyclonal	200µg Liquid form	Abcam	1/200(ELISA) 1/100 (IHC-P)

Table 3.2: Secondary Antibody

Name	Description	Concentration	Producer	Dilution
Ab6721	Goat anti-rabbit IgG H&L (HRP)	1 mg Liquid form	Abcam	1/120000(ELISA) 1/500 (IHC-P)

3.7.2 Solutions Preparation

3.7.3 Blocking Solution 1% BSA

Bovine serum albumin (BSA) (HyClone) was used as a blocking serum to block the residual protein binding sites and prevent false positive results due to the cross-reaction of antibodies or non-specific binding with the antibodies, and to improve the sensitivity

of the test by reducing background interference. 5g of powdered BSA was added to 500ml of PBS without stirring or mixing and placed at 4°C for 30 mins. After 30 mins the BSA powder was dissolved and the liquid was then aliquoted and stored at -20°C.

3.7.4 Hematoxylin and Eosin (H&E) Staining Protocol

The sections were deparaffinized at two changes of xylene for 5 mins each, rehydrated in absolute alcohol (100%) for 3 mins, 95% alcohol for 3 mins, and 70% alcohol for 3 mins. Next, the slides were washed for 3 mins under running water. The slides were then dipped in hematoxylin solution and left for about 5 mins. They were then washed under running water for another 5 mins. For differentiation, 0.5% acid alcohol was used for 10 seconds to remove any excess background staining and produce definitive nuclei with crisp detail, followed by washing under running water immediately. For blue or purple chromogenic stain of the nucleus, 2% sodium acetate buffer was used as a bluing reagent. The slides were dipped in this alkaline buffer four times then placed under running water for 3 mins. The slides were then rinsed in 80% alcohol for 1 min and counter-stained in eosin solution for 2-3 mins. The slides were dehydrated through 95% alcohol, four dips, and two changes, two changes in absolute alcohol for 2 mins each, and clearing in 3 changes of xylene, 3 mins each.

3.7.5 Immunohistochemistry Procedure (IHC)

i. Preparation of the slides

The immunohistochemistry protocol used in this study was according to (Giribabu et al., 2017). The slides were incubated at 37°C overnight followed by incubation at 60°C for one hour to prevent the detachment of tissue from the slides (Figure 3.1).

ii. Dewaxing

The slides were dewaxed and deparaffinized in 3 changes of xylene each for 5 minutes (xylene I, II and III), washed in 100% ethanol twice for 10 minutes each, and in 95% ethanol twice for 10 minutes each. Next the slides were washed with PBS 2 times for 2 minutes, then washed in ddH_2O for 2 minutes while stirring. The excess liquid was aspirated from the slides.

Figure 3.1: (Memmert Incubator, Germany) used in the experiment to prepare the slides before running the actual IHC protocol

*From this point the slides have not been allowed to dry to prevent high background staining resulting from non-specific binding of antibodies.

iii. Heat-induced Antigen Retrieval (scientific microwave)

During the fixation process, preservation of peptides and cellular organelles occurs by binding of formalin with uncharged reactive amino groups (-NH or NH2) of amino acids. The end result of the reaction is profound changes that interfere with the immune-detection of antigens. Yet exposing the tissue sections to antigen retrieval before immunostaining is mandatory to unmask the antigen sites by breaking the methylene bridges and exposing antigenic sites. Moreover, this process will facilitate the antigen-

antibody binding. This revolutionary technique was attempted with varied methods such as enzymatic, non-heat pretreatment methods. In 1991 Shi et al, reported for the first time using heat-induced antigen retriever. The original heat source was a microwave, with time pressure cookers coming in place. Others had used autoclaves, water baths, and steamers. However, in this study a microwave was used as the heat source.

Figure 3.2: The scientific microwave processor (Labpulse 2850), used in the experiment for heat induced antigen retrieval

iv. Citrate Buffer Solution preparation at pH 6.0 and 0.01M

2.94g of trisodium citrate was dissolved in 800ml of distillated water. After ensuring the complete dissolving of the salt, the pH of the solution was adjusted to 6.0 by adding drops of HCL. The final volume of distillated water was adjusted to reach 1L. Then, 500 µl of tween-20 was added.

v. Procedure

The slides in the slide holder were dipped into antigen retrieval solution, boiled in a scientific microwave at 50°C for 20 mins and then at 90°C for another 20 mins. The slides were then allowed to cool in buffer for 20-30 minutes, then washed in deionized

water for 2 minutes and cleaned with tissue paper then kept in a cassette in a petri dish (Figure 3.2).

vi. Hydrogen Peroxide

To prevent false positive staining and high background signal resulting from interaction between endogenous peroxidases and substrate solution, the samples were pre-treated with hydrogen peroxide (H_2O_2) before incubation with HRP-conjugated antibody.

One drop of DaKo Real peroxidase – blocking solution was added to the slides and incubated for 10 minutes. Then, the slides were washed with PBS for 5 minutes (2 times).

vii. Blocking Step

1% BSA (HyClone) was used as a blocking serum. The solution was made by adding 5g of lyophilised BSA powder to 500 ml of PBS without mixing the solution to prevent foam formation. The mixture was then placed at 4°C for 30 mins until the powder dissolved. Aliquots were stored at -20°C.

For each slide 200 µl of 1 % blocking serum was added and kept for 1 hour.

The slides were not rinsed (only drained) and wiped around the sides by tissue paper.

viii. Primary Antibody.

The slides were incubated with the following diluted primary antibodies overnight at ≈90% humidity and 4°C: rabbit monoclonal anti-IL-1RA antibody (ab124962; Abcam, Cambridge, U.K.), rabbit polyclonal anti-IL-8 antibody (ab7747; Abcam, Cambridge,

U.K.), rabbit polyclonal anti-IL-6 antibody (ab208113; Abcam, Cambridge, U.K.), and rabbit polyclonal anti-TNF-α (ab6671; Abcam, Cambridge, U.K.). Each of the primary antibodies were diluted according to the dilution factors as recommended by the manufacturer to help in identification of the antigen and to increase the chance for specific binding of the antibody to the target antigens. All dilutions were prepared in 1.5ml Eppendorf tubes (Table 3. 3).

Table 3.3: Antibody Dilution

Antibody	Dilution Factor
IL-8	1/100
IL-1	1/100
TNF-α	1/100
IL-6	1/50

When the antibody dilution for each of the primary antibody has been established, 200 μm of the diluted antibody was then applied to the formula (refer to equation 3.2).

$$200\ \mu m\ \times number\ of\ slides = (total\ volume)\mu l \qquad (3.2)$$

$$\frac{(total\ volume)\mu l}{dilution\ factor} = (final\ volume)\mu l$$

ix. Secondary Antibody.

The slides were incubated with diluted secondary antibody, goat anti-rabbit IgG (HRP) (Ab6721; Abcam, Cambridge, U.K.) for 2 hours at room temperature and ≈90% humidity. After two hours the slides were kept in containers, deionized water was poured over and the slides were washed (by gently shaking) 3 times for 5 minutes.

Table 3.4: Secondary Antibody Dilution

Antibody	Dilution Factor
HRP	1/500

The dilution was calculated according to the following formula ((refer to equation 3.3):

$$200\mu m \times number\ of\ slides = (total\ volume)\mu l \qquad (3.3)$$

$$\frac{(total\ volume)\mu l}{dilution\ factor} = (final\ volume)\mu l$$

x. DAB-Peroxidase Substrate Solution (Brown)

DAB–peroxidase (Dako liquid DAB + substrate Chromogen system) was used in this study for immunohistochemistry staining**.** Tissue sections were incubated at room temperature for up to 10 mins until suitable staining developed. In one tube 2ml of ddH_2O was added with 1 drop of buffer solution, 1 drop of DAB stock solution and 1 drop of substrate reagent. After the stain developed, the reaction was stopped by washing the slides with ddH_2O for 5 mins after the stain (one time only) (Figure 3.3).

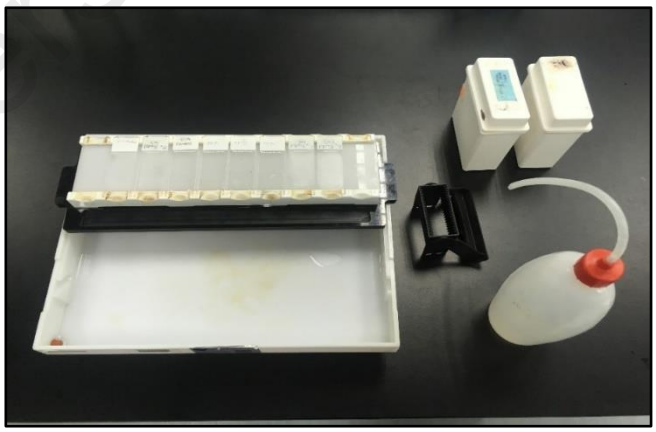


Figure 3.3: The image shows the process of IHC procedure on skin rat sample from the frontal region to detect the inflammatory biomarkers

xi. Counterstain with Hematoxylin

Hematoxylin was used to contrast the brown color of the DAB stain and make the stained nuclear structure clear under the microscope. The slides were sucked for 20 seconds then washed with distilled water 3 times.

xii. Rehydration

The slides were rehydrated in 95% ethanol for 10 seconds two times and washed in 99% ethanol for 10 seconds two times. Then, dipped in xylene 1 for 10 seconds, xylene 2 for 10 seconds, and xylene 3 for 10 seconds as well. The coverslips were mounted over the slides by using (Leica Biosystems) mounting medium.

xiii. Negative tissue control

Negative controls were established by omission of the primary antibody. The control tissue slides were incubated with PBS instead of primary antibody followed by incubation with secondary antibody.

xiv. Visualization of the histological specimens

All the slides were digitized with the Panoramic SCAN digital slide scanner (3DHISTECH, Budapest, Hungary). The histological images were subsequently analyzed by panoramic case viewer software version 1.15.3 (3DHISTECH, Budapest, Hungary) at 40X magnification.

xv. Scoring of immunohistologically positive stained samples

The presences of immune reactive cells were analysed and scored based on a semi-quantitative scoring system using panoramic case viewer software version 1.15.2 (3DHISTECH, Budapest, Hungary). Each slide was divided into nine equal targeting areas about $4\times8\mu m^2$ in size, three targeting areas for each layer of skin: epidermis, dermis, and hypoderm. The positive and negative cells were then counted at 40 times

magnification. The total percentage of the positive cells was calculated by dividing the number of positive cells by total cell number and multiplying by 100, similarly for the total negative cell percentage, and the scores were classified as 0 = negative; weak ≤ 10%; moderate = 11-50%; strong = 51-80%, and very strong = ≥ 80% (Fedchenko & Reifenrath, 2014).

3.8 Statistical Analysis

The distribution of the data was analysed by Shapiro-Wilk test for the normality of the datasets, the data were not normally distributed. Comparison between control and expanded groups was then performed by using the non-parametric Two- independent test (Mann-Whitney U test). The data were positively skewed due to the large number of (0) values within the variance, thus the mean will not be a good representation of the data. Instead, the data was presented with the mean rank of the mentioned test (Milenovic, 2011). The p-value generated from the statistical test was used to demonstrate the significant difference between the control and expanded tissues, considering significance at $p \leq 0.05$, and respect to a two-tailed probability distribution. The non-parametric Spearman's correlation coefficient was used to evaluate whether the immunepositive expanded tissues were correlated with the inflammatory cells in the same particular tissue, and the (2-tailed) p-value was used to assess the significance of the correlation. All the analyses were performed by Statistical Package for the Social Sciences (SPSS) version 21.

CHAPTER 4

RESULTS

4.1 The effect of anisotropic hydrogel tissue expander on skin histology

H&E stain assessed the effect of anisotropic hydrogel tissue expander on skin tissue histology and morphology. A standard light microscope examination of tissue specimens at four weeks post-expansion showed that the epidermis of the expanded tissues were slightly thinner than those in controlled tissues. In the expanded tissue the cells consisted of one single lining layer and were scattered randomly. In contrast, the cells of the control samples were composed of three lining layers and maintained their architecture and were still intact. In terms of the shape however, the expanded epidermal cells were more flattened compared to the cuboidal shape of epidermis cells in control samples. The dermis in expanded skin was increased in terms of thickness compared to the control an increase in the number of fibroblasts was observed, in reticular dermis the collagen fibers were arranged in an irregular manner. In the tissue sections the dermal papilla appeared normal, the blood vessels appeared with thinning and diluted walls (Figure 4.1, Figure 4.2).

4.2 The effect of anisotropic hydrogel tissue expander on inflammation

The dermal papilla appears normal. Taken together in the dermis layer, the tissue section shows no inflammatory cell invasion absence of polymorphonuclear cells and other WBC around the dermal blood vessels, and a decent number of resident neutrophils and eosinophils present at the dermal layer (Figure 4.3).

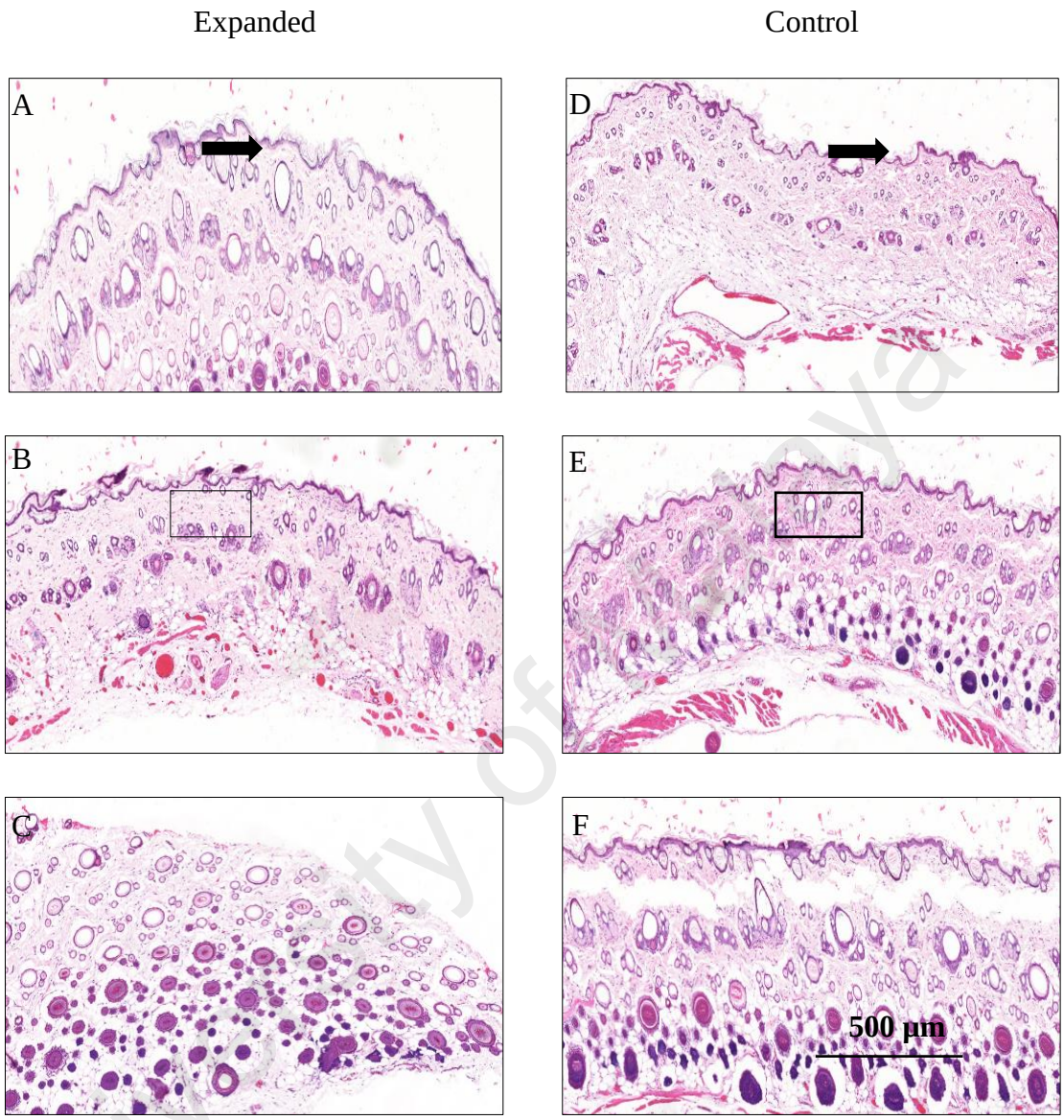


Figure 4.1: H&E staining of rat skin. The expanded tissue cells were thinner, flat and scattered (A, B, C) (as shown by the arrows) when compared to the cells of the epidermis layers in the control groups that were thicker with cellular architecture maintained (D, E, F).

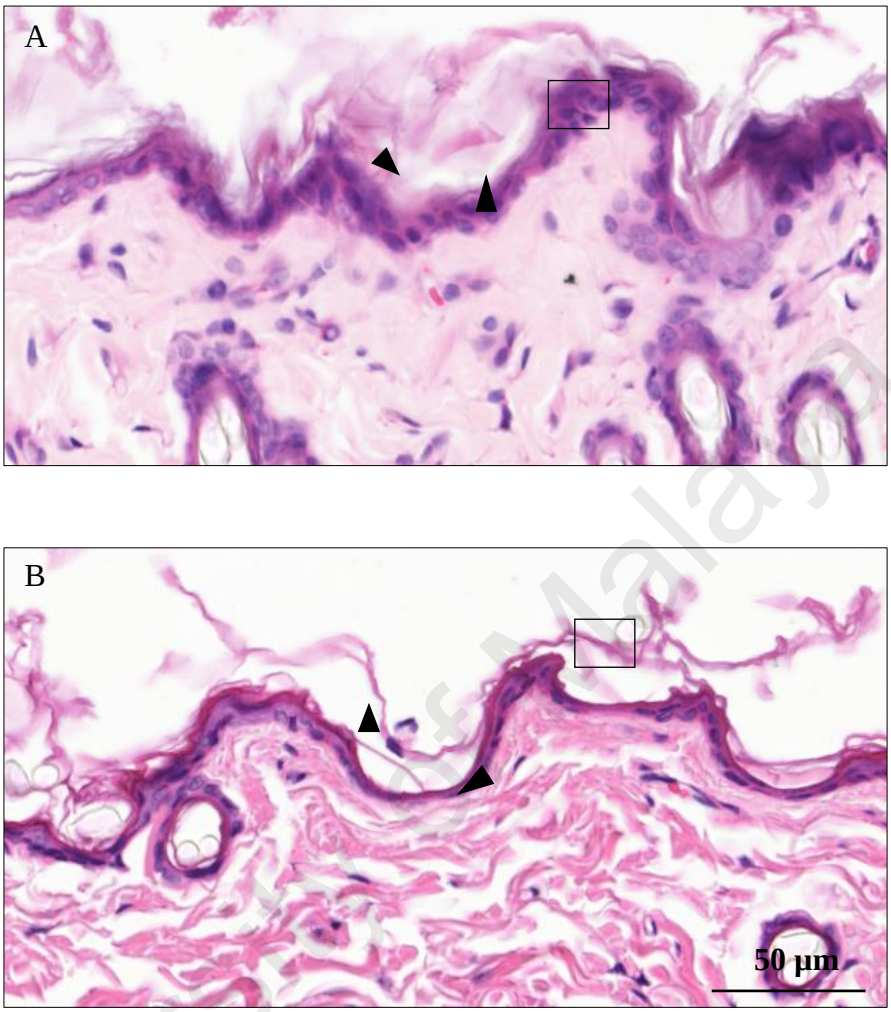


Figure 4.2: H&E. The epidermis (shown in boxes) of control skin (A) consists of; two to three layers of epithelial cells and the epidermis of the expanded skin tissue (B) consists of single, flattened cells. The dermal collagen fibers (black triangles) are scattered and irregular with eosinophilic matrix. The fibers appeared fragmented in expanded sections compared with the dermal collagen fibers in the control sample.

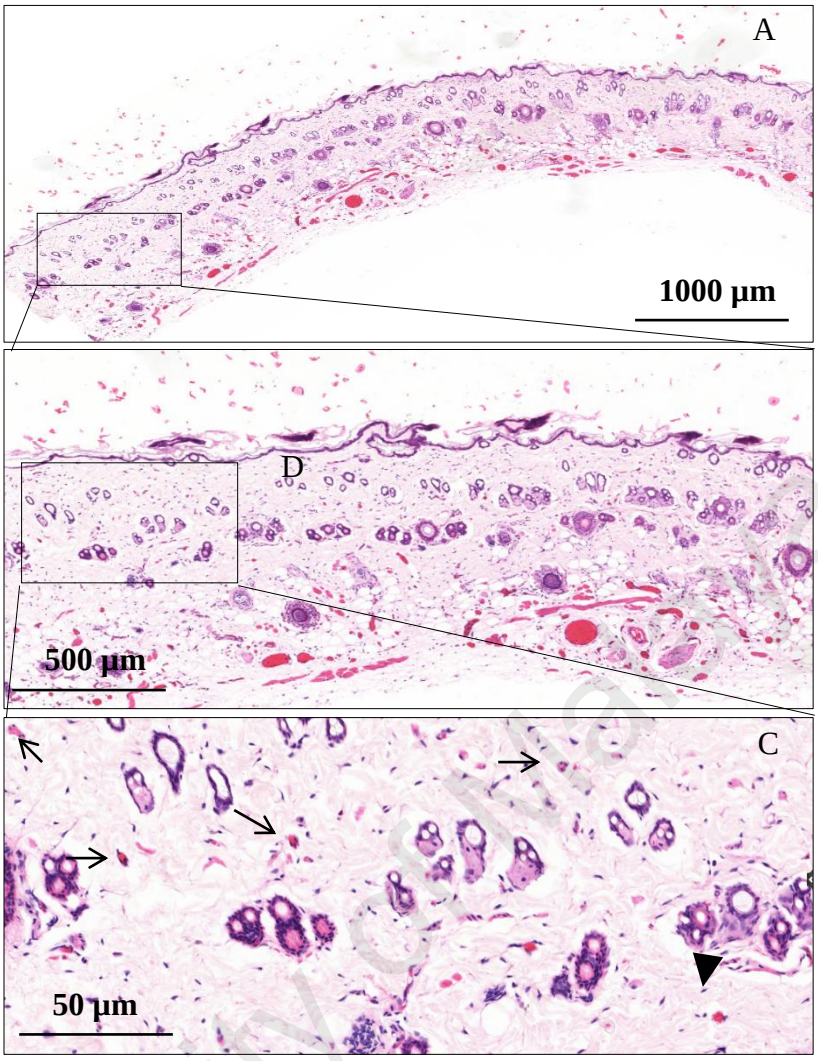


Figure 4.3: Histology of the scalp skin of rat after 4 weeks post-expansion: H&E staining section of expanded tissue where (A), (B), and (C) represent different magnification of the dermis, and shows absence of inflammatory infiltrations. However, isolated resident neutrophils (shown with black arrow) and eosinophils (shown by black triangle) are present at the connective tissue

4.3 Immunohistochemical findings

4.4 The effect of anisotropic hydrogel tissue expansion on pro-inflammatory cytokine and chemokine production

4.4.1 Expression of IL-1

The immunohistochemical section indicated that the content of IL-1 was very strong in the expanded tissue, about 83% compared to 0% in non-expanded tissue. About 91% of keratinocytes were immune-active and IL-1 was present intracellularly in the epidermis layer. Moreover, in the dermis about 97% of the cells strongly expressed IL-1

in the nucleus and in the cytoplasm, while the majority was at the glassy membrane and the medulla of the hair follicles. The cells of the pilosebaceous glands and their ducts showed the highest level of the target protein. Additionally, all the collagen fibers in the dermal layer were immune-positive compared to the static control. In the hypoderm only the endothelial cells were immune-positive, around 64% (Figure 4.4 and Figure 4.5).

Expansion enhances IL-1 secretion by epithelial cells, as seen in (figure 4.6) which represents compression between expanded and non-expanded tissue.

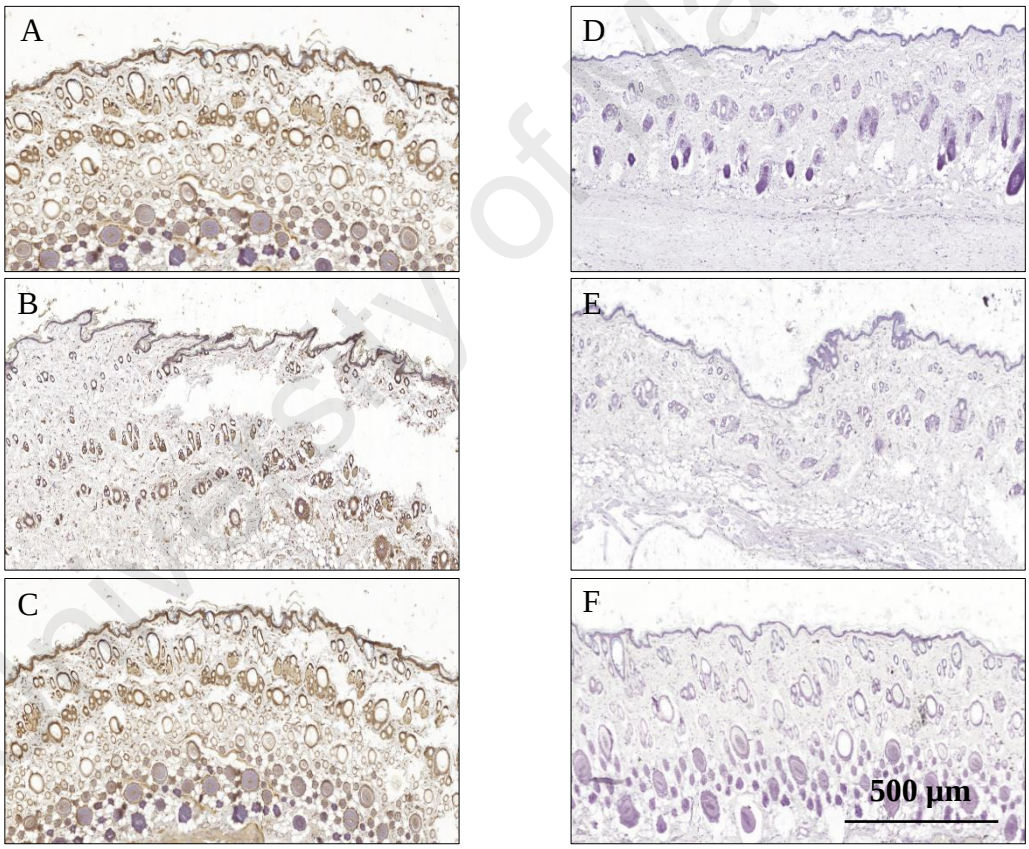


Figure 4.4: Figures depict six histological slides from different rats: (A, B, C) expanded groups, and (D, E, F) control groups. The epidermis, dermis, and hypodermis cells of the expanded groups were immune-positive to anti-IL-1 antibody, and showed nuclear and cytoplasmic expression. In contrast, all the epithelial cell layers of control group were immune-negative to anti IL-1.

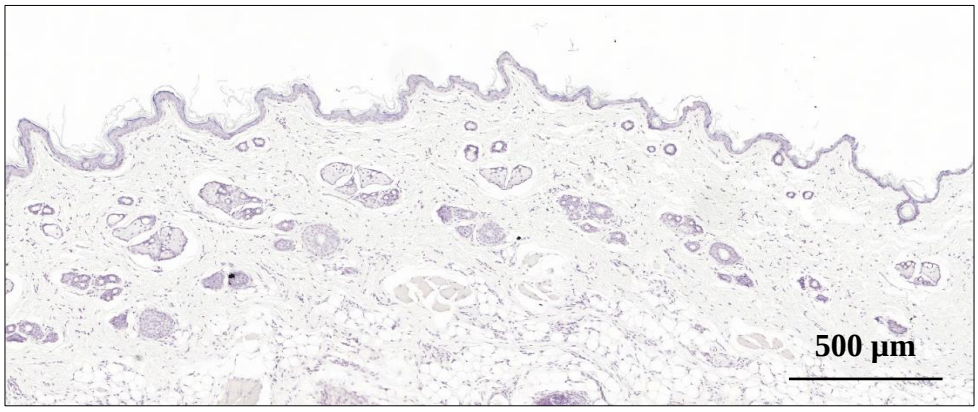


Figure 4.5:-Representative histological slide of negative control for IL-1. The process was carried out by omitting the primary antibody. The epithelium cells were negatively stained similar to control samples

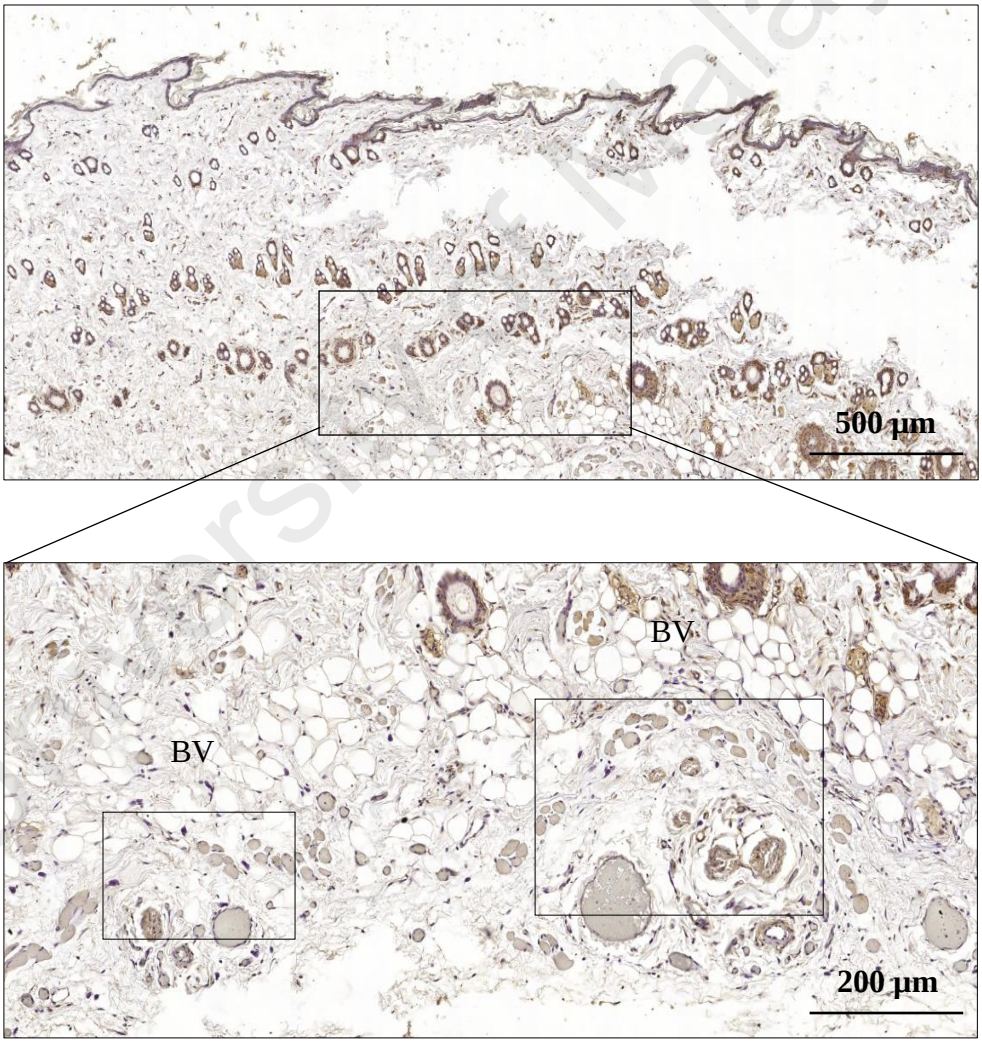


Figure 4.6: Rat skin specimens of frontal region were harvested for immunohistochemistry and show strong positive endothelium cells in the hypodermis layer. (BV= Blood Vessels)

4.4.2 Expression of IL-8-, IL-6, and TNF-α

IL-8, IL-6, and TNF-α expression was not affected by the stretching force generated by hydrogel tissue expansion, as 100% of the cells were immune-negative, similar to the control samples (Figure 4.7, Figure 4.9, and Figure 4.11) and the negative control (Figure 4.8, Figure 4.10, Figure 4.12) samples respectively.

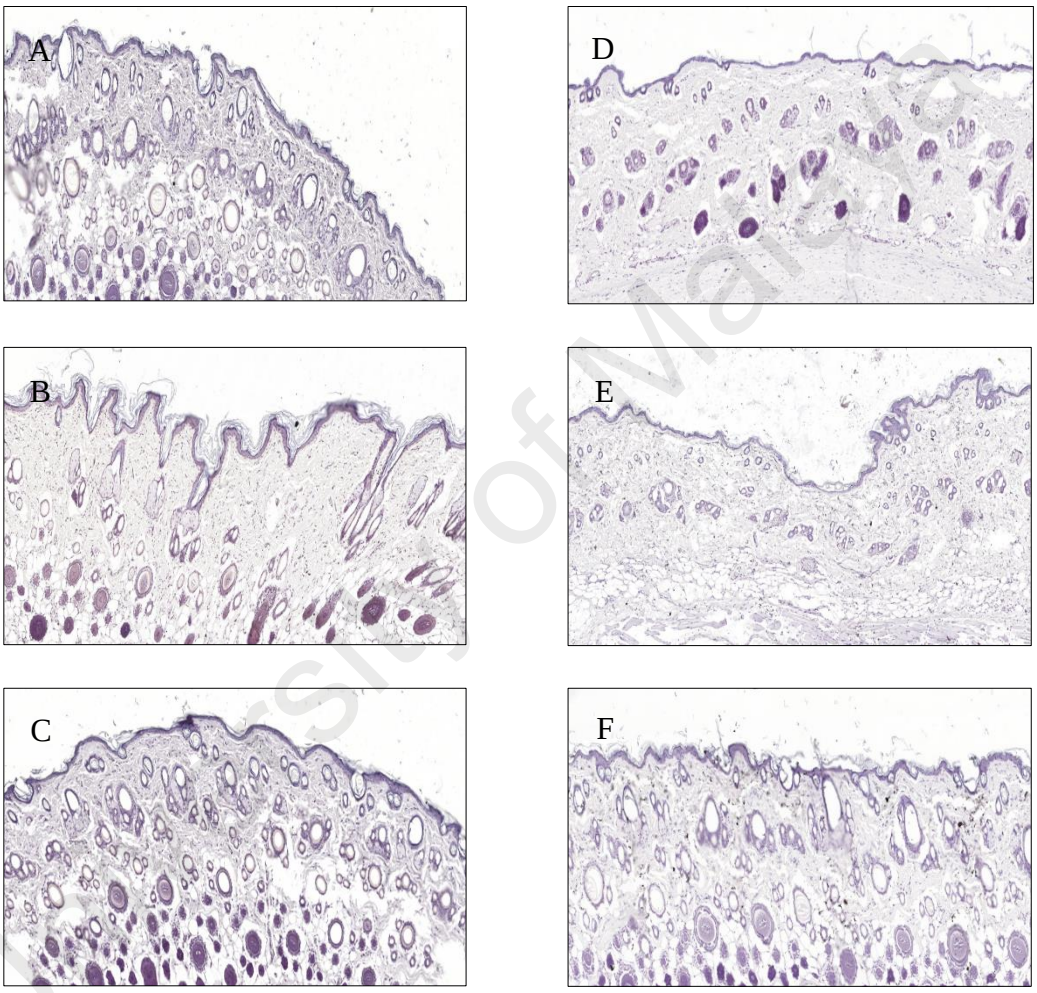


Figure 4.7: Histological section of rat skin tissue. Representative images of immunohistochemical stain of IL-8. The images show that all cells were immune-negative in the expanded tissue (A, B, C), similar to that in the control tissue (D, E, F)

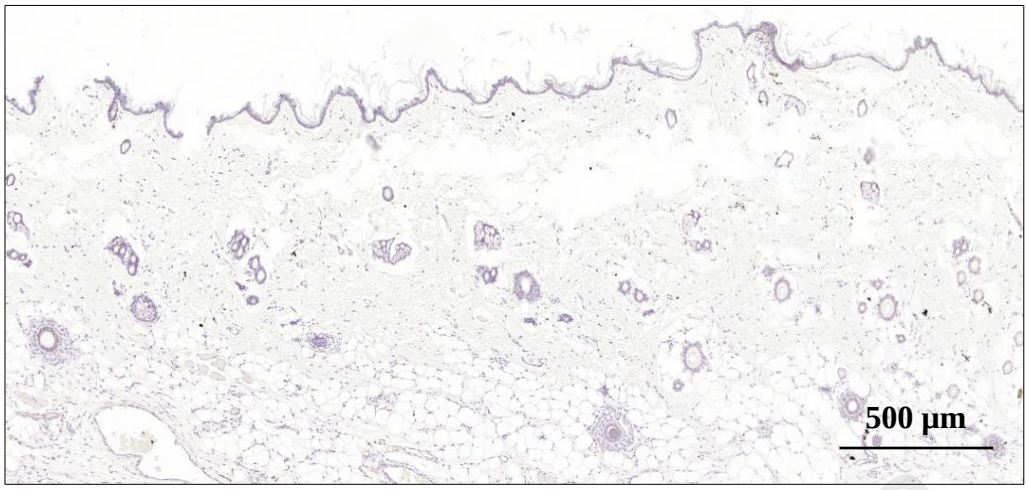


Figure 4.8: Histological image of a negative control tissue to IL-8 antibody. The procedure was carried out by omitting the primary antibody

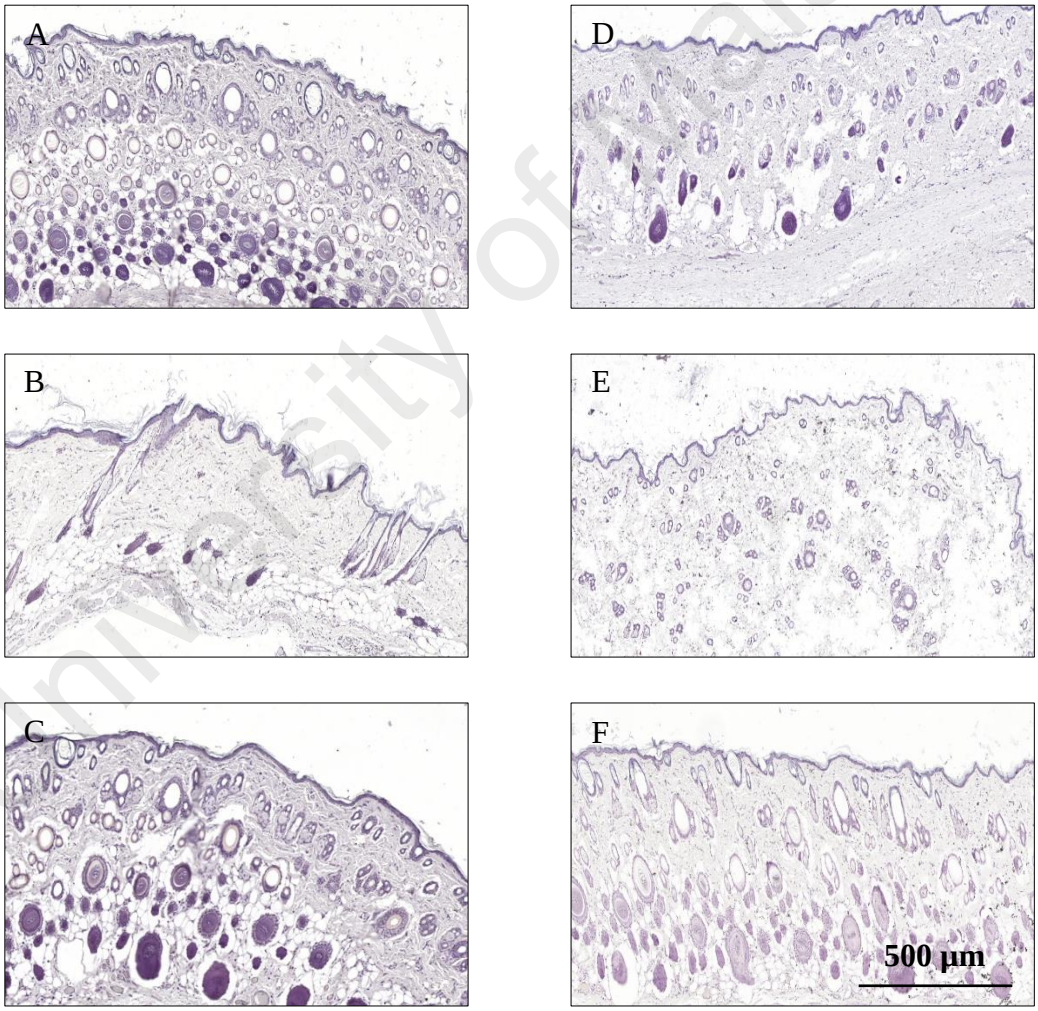


Figure 4.9: Representative images of immunohistochemical stain of IL-6. Immune-negative cells in expanded tissue (A, B, C), and the control tissue (D, E, F)

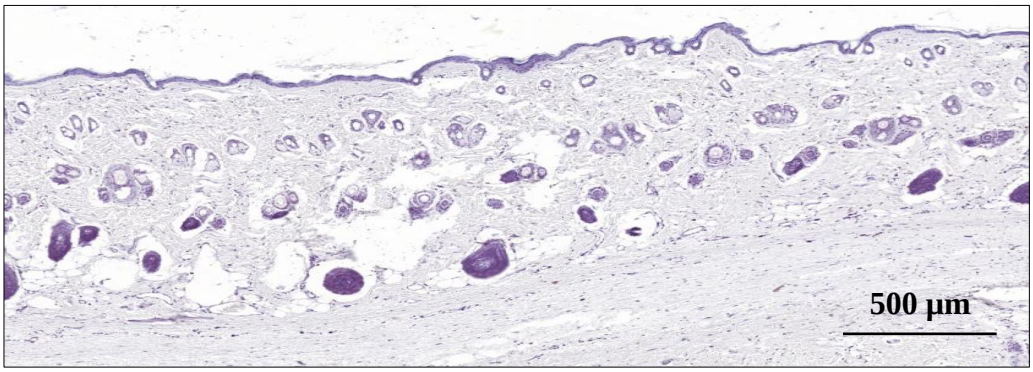


Figure 4.10: Negative control for IL-6. The procedure was carried out by replacing the primary antibody with phosphate buffer saline solution

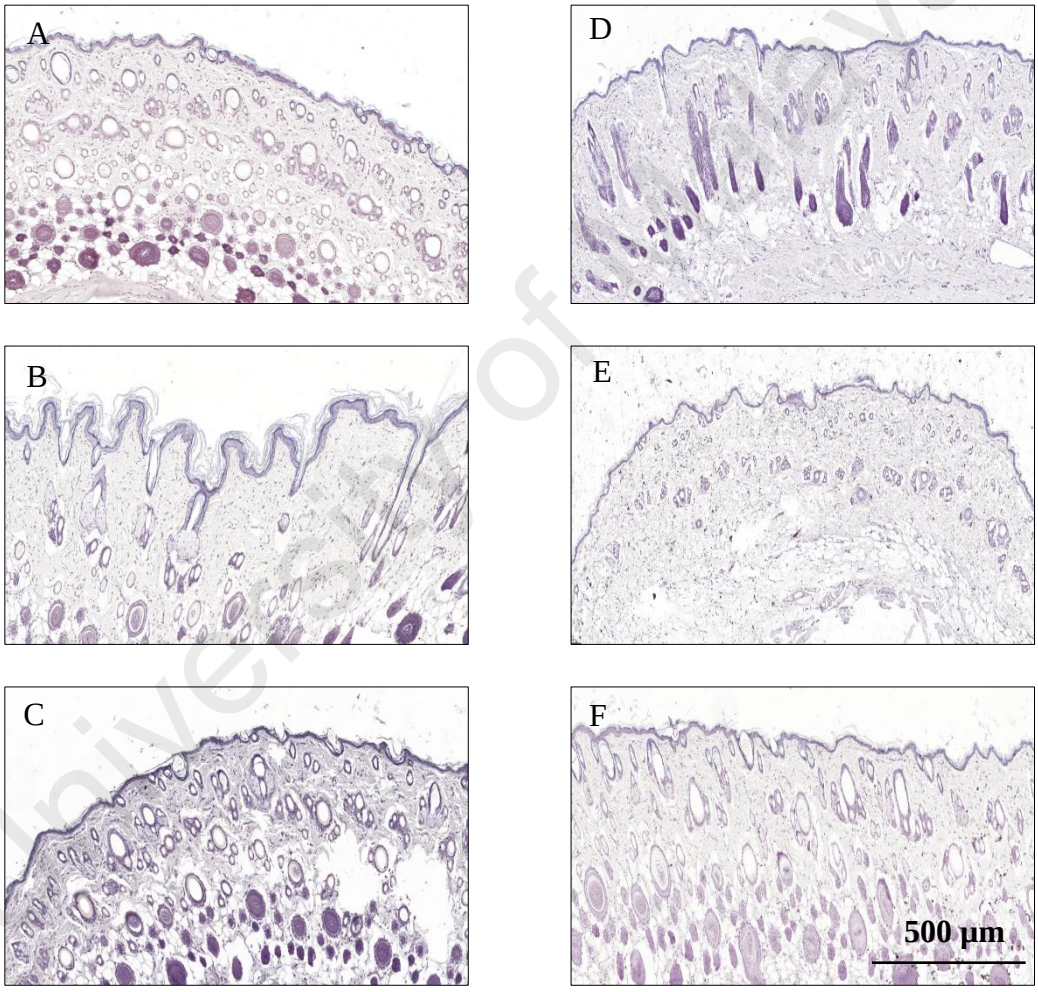


Figure 4.11: Representative images of immunohistochemical stain of TNF-α. immune-negative cells in expanded tissue (A, B, C), which are similar to control tissue (D, E, F).

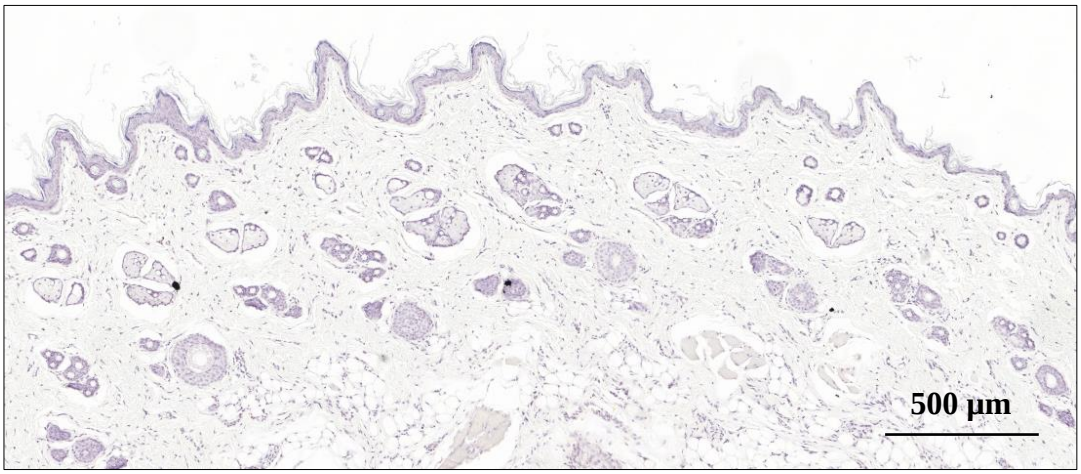


Figure 4.12: Negative control of TNF-α

4.5 The difference between dytokine droduction in dxpanded and non-expanded skin tissue samples

The production of pro-inflammatory cytokines was higher in the expanded samples with a mean rank of 14.00 compared to the mean rank of control which was 11.00. However, the different mean rank of cytokine expression between the two groups appears not to be significant ($p = 0.071$).

4.6 The association between inflammatory cell profiles and cytokine production in immune-positive expanded tissue

There was a weak negative correlation between the immune-positive expanded samples and the presence of the resident inflammatory cells neutrophils and eosinophils associated with acute inflammation, with a Spearman's correlation coefficient of $r = -0.500$. However, this negative correlation statistically was not significant as the sig (2-tailed) p-value was 0.667 which is more than 0.05, thus the reduction of one variable does not indicate an increase in the other.

CHAPTER 5

DISCUSSION

Clinical soft tissue expansion has become revolution in the last three decades where it relies on the ability of the tissue to remodel and growth when mechanical force is applied. It is an ideal way to attain extra tissue which remarkably matches in color and texture with the surrounding healthy tissue. Application of tissue expansion had reduced the common complication associated with flap procedure to close large defect. It was first presented by Neumann in 1957 to reconstruct injured traumatic ear using rubber balloon filled with air (Nuemann, 1957). This method then spreads worldwide and become acceptable after it was proposed by Radovan and Argenta in 1980s (Radovan, 1982, 1984) to restore breast deformity after mastectomy in breast cancer patients. Austad and Rose came up with a new self-inflating device based on balloon made of semipermeable membrane of silicon which contains hypertonic and highly saturated sodium chloride solution. This model expands by drawing the extracellular tissue fluid by osmotic forces (Austad & Rose, 1982), Solution related problems were observed and caused necrosis at the adjacent tissue. As a result, the clinician abandons this type of self-inflating expander. The basic concept was revived again in 1993 by Wiese in Germany when he presented a model which consisted of modified copolymer hydrophilic hydrogel to be used as a self-inflating expanders (Wiese, 1993) Hydrogels are macromolecule with cross-link networks that are capable to take in water and swell in size. The network permits the passage of liquid molecules and keeps out other dissolved material and acts as a barrier. Wide ranges of hydrophilic polymers have been quantified, but only p (AMM-NVP) based self-inflated expanders is widely and clinically used. Despite its popularity, there is a lack of quantitative understanding of how does the tissue response to it. Generally, when the skin is stretched, new skin is

created and changes in tissue microstructure occur, activation of stress-induced signaling pathways causes multiple molecules to be secreted in response to the stress to control cellular function, division and proliferation or death. Nevertheless, immune cell activation and attraction to the surgical site are the initial response of our body to the implanted material in general by secretion of several active biological molecules such as, enzymes, lysosomes and cytokines.

In the present study, the effects of anisotropic hydrogel tissue expander were evaluated as an inflammatory trigger of proinflammatory cytokines. However, our findings show that anisotropic hydrogel tissue expander had no significant effect on targeting the immunological pathways. This agrees with the hypothesis and with the clinical results of previous studies as mentioned in the literature review. The expansion rate of the device was as expected and post-expansion problems such as tissue necrosis, cellular degeneration and sub-clinical inflammation were not detected histologically.

On the other hand, its effect on the immune system and inflammatory signaling has yet to be explored. Numerous studies demonstrated that tissue expansion in general would lead to proinflammatory cytokines (Vlahakis et al., 1999) but the specific triggers for those inflammatory cytokines and cellular origins of inflammatory mediators are still unclear. We chose to investigate IL-1, IL-8, TNF-α, and IL-6 as markers in this study because they are the primary proinflammatory mediators involved in the inflammatory process (Ahmad et al., 2018; Cuomo et al., 2018).

Although previous studies show no clinical manifestation of inflammation, the presence of IL-1 in the expanded tissue indicated that despite the absence of inflammation there are other factors which stimulate its secretion. Activation of different key modulator proteins will lead to a different inflammatory response

pathway. An increase or decrease in inflammation will induce the transcription of genes that provoke cellular growth (Buhrmann et al., 2018; Chai et al., 2015; Jetten et al., 2013; Porta et al., 2009; Scholz & Taylor, 2013).

Generally, TNF-α has been always case dependent and linked with IL-1 but in this study, it was not discussed. . The presence of both IL-1 and TNF-α is a strong indication of inflammation, and this does not agree with the clinical finding. Moreover, TNF-α in the skin is produced mainly by activation of resident dermal mast cells (Shirley et al., 2016). These cells contain numerous sizable active TNF-α inside their granules which can be rapidly released to the extracellular spaces upon degradation of the cells and these cells are found to be the only source of TNF-α located near the blood vessel, Thus, in H&E stained section, there was no sign of scattered mast cells, apoptotic cells or degranulated cells. Additionally, in the inflammatory statues, Elevation of IL-6 has direct relation with IL-1 and TNF-α. The combination of these three cytokines had been recognized as targets of therapeutic intervention of inflammation for many years (Scheller et al., 2011). Furthermore, as it has been reported in studies that apoptosis induces IL-6 (Chalaris et al., 2007), it is reasonable that we did not detect IL-6 immune active cells in our expanded tissue.

Il-8 is a powerful chemoattractant for neutrophils due to its small molecular mass (less then 10KDa) and it facilitates the migration of neutrophils towards the endothelial cells. It is produced by non-inflammatory cells such as fibroblasts and epithelial cells upon the initial host response to any stimuli (Ishiko et al., 2003). IL-8 stimulates granulocytes to release a toxic byproduct of oxygen which causes oxidative stress in cellular DNA and other proteins. In H&E staining there was no extravasation of leukocyte migration. However, both the superficial and deep blood vessels were diluted despite the absence of inflammatory cells.

When mechanotransduction force is applied on the skin by tissue expander, cellular structure and cytoskeletal network architecture are affected. However, even though some changes are reversible after removing the expander, new tissue growth resulting from tissue creep is indeed irreversible. Dermal stretch beyond its physiological level triggers multiple different stress-induced signaling pathways (Lee et al., 2018) resulting in cellular growth activation, cytoskeletal proliferation, and increase in neovascularization in the dermal cell layer (Tepole et al., 2011). Most of the dermis is made of collagen (Aziz et al., 2018), making up 70% to 90% of its dry content, giving it structure and strength (Khavkin & Ellis, 2011; Papadopoulou et al., 2016). During active tissue expansion, fibroblast cells increase in number and mitotic activity, thus increasing extracellular matrix protein and collagen deposition. Most of the data from previous studies provide information only at macroscopic level. Further experiments are required at the cellular level to clarify the biological pathways involved in the growth process of fibroblasts in relation to tissue expansion. However, remarkable recent studies have found that IL-1 plays a crucial role in inducing and/or increasing fibroblast collagen synthesis *in vitro* (Goumans et al., 2014; Ricard et al., 2018). It was found that IL-1 is a cross-talk signaling molecule from epithelium cells to fibroblast cells in normal immune defense (Osei et al., 2016). In addition, a recent study found that the expression of osteopontin (OPN) which is a pro-fibrotic molecule, is directly increased by IL-1 (Shimodaira et al., 2018). In skin dermis, production of collagen by fibroblasts is down-regulated by keratinocytes via production of IL-1 (Harrison et al., 2006; Sun et al., 2017). It is noteworthy that IL-1 down-regulates angiogenesis. Presence of IL-1 immune-positive endothelial cells in our samples indicate that the cell might produce it in response to expansion as a protective mechanism to prevent abnormal proliferation and tumor formation due to anti-angiogenic function of IL-1 (Ribatti, 2018). As a general rule, tissue expansion does stimulate the formation of new blood vessels.

Studies found that IL-1 directly stimulates angiogenesis via vascular endothelial growth factor (VEGF) signaling pathway. This pathway is crucial in normal physiological angiogenesis and wound healing. In contrast to IL-6, it stimulates angiogenesis via two pathways, NOTCH ligand jagged pathway (Gopinathan et al., 2015) and angiopoietin–tie pathway, as those two are considered to be the main pathways involved during pathological vascular remodeling and tumor angiogenesis (Saharinen et al., 2017). Thus, absence of IL-6 in our samples may be a good indicator for physiological angiogenesis. Moreover, VEGF does result in spontaneous epithelization and collagen deposition (Bao et al., 2009). All of the mentioned events may explain the reason behind finding IL-1 in our study. Furthermore, histologically dense dermal layer of the expanded tissue may indicate active collagen deposition and abundant vascularization. IL-1 alpha play an important role in keratinocyte differentiation and proliferation by increasing the secretion of integrins in the basal and suprabasal cell layer, integrins are a group of transmembrane receptors that function mechanically by facilitating the adhesion of a cell to the extracellular matrix during wound healing (Hobbs & Watt, 2003).

On the other hand, recent analysis demonstrated that increased production of IL-1 indeed induced a state of pathological collagen deposition in progressive fibrotic diseases such as liver, kidney and skin (Artlett, 2017; Park et al., 2018). Similarly, increase in activation of IL-1 stimulate body cells to express fibroblast growth factor 2 leading to cell migration (Lee & Heur, 2013).

To the best of our knowledge, this is the first *in vivo* study yielding useful information on how tissue responds to novel anisotropic self-inflating hydrogel tissue expander from a macro-molecular point of view with regards to inflammation.

However, what could be the possible cause of cellular expression of IL-1 remains a topic of further investigation.

CHAPTER 6

CONCLUSION

6.1 Summary

The effect of anisotropic self-inflating hydrogel tissue expander on inflammatory response in skin was investigated using histology and immunohistochemistry in this study and the following conclusions were obtained:

i. The data of this study support the concept that the anisotropic hydrogel tissue expander does not promote subclinical activation of the immune system involved in the inflammation.
ii. The data obtained further highlight the molecular aspect of it.
iii. It was demonstrated that most of the investigated IL-6, IL-8, TNF alpha proinflammatory biomarkers were not present in the expanded skin tissue. However, IL-1 cytokine in expanded tissue was very strong, about 83% compared to 0% in controlled.
iv. Presence of IL-1 in expanded tissue with the absence of inflammation assumed that other factors contribute to its formation.
v. Histology shows thinning on the epidermis and thickness of the dermal cell layer after the expansion.
vi. There was no inflammatory reaction on the expanded tissue after 4 weeks of expansion.

6.2 Recommendations and Future Suggestion

Further macromolecular studies are recommended to be conducted to study the correlation between inflammatory biomarkers and anisotropic self-inflating hydrogel tissue expansion. Future studies can be carried out to compare the soft tissue reaction in relation to slow and rapid expansion.

Useful future study will undoubtedly help to understand the effect of anisotropic self-inflating hydrogel tissue expansion on fibroblast cells proliferation and collagen formation, and whether this activation require the presence of IL-1 as the fibroblast potentially can be considered as promising cells for tissue engineering and has been applied widely in regenerative medicine in the last decade. A large component of the tissues are composed of fibroblast and their mesenchyme origin makes them suitable for tissue engineering either by development of the desired tissue or to improve stem cells based knowledge and technology. Additionally, it can also be investigated with a comparative study between slow and rapid expansion. Moreover, studying the interaction between tissue expansion and different signaling pathways is expected to reveal how crucial multifactorial IL-1 was secreted, and to determine its source, kinetic production and regulation.

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