THE INFLUENCE OF ANISOTROPIC TISSUE EXPANDER ON NESTIN AND TRKB EXPRESSION IN RAT CRANIO-FACIAL REGION.

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ABSTRACT

THE INFLUENCE OF ANISOTROPIC TISSUE EXPANDER ON NESTIN AND TRKB EXPRESSION IN RAT CRANIO-FACIAL REGION.

Introduction: Skin is a highly dynamic, autoregulated, living system that responds to mechanical stretch providing a net gain in skin surface area. The techniques for expanding skin and soft tissues are widely used to repair damaged tissues and to facilitate the provision of new additional skin tissue with similar quality, texture and color. Little is known regarding the effect of tissue expansion on peripheral nerve. Should the new nerve could be generated by this method without significant damage then the additional skin generated may present with the similar sensation as the adjacent skin.

Objective: (1) To compare the histological changes between non-expanded and expanded tissues in a rat model implanted with anisotropic tissue expander. (2) To investigate the presence of nestin and TrkB protein marker expression in expanded and non-expanded tissues.

Method: Twenty adult female Sprague Dawley rats 180-220 g were used in this study. Ten anisotropic controlled rate self-inflating tissue expanders (Oxtex, UK) were implanted subcutaneously at the scalp region under general anaethesia. After 28 days, animals were euthanized, the expanders were explanted and expanded skin were harvested. Immunohistochemistry was performed to detect the presence of nestin (Rat 401) Mouse mAb) (CST) and TrkB (NT-3) expression. (CST).

Results: Nestin immune-positive staining was found on the expanded skin, while TrkB expression was comparable to the normal skin.

Conclusion: Tissue expansion using anisotropic self-inflating tissue expander resulted in expansion of the skin tissue with possibility of similar innervation and histological texture that resembles the control tissue.

Keywords: peripheral nerve, Nestin, TrkB, anisotropic tissue expander, epidermis, dermis.

ABSTRAK

PENGARUH PENGEMBANGAN TISU ANISOTROPIK DI BAHAGIAN KRANIO-FASIAL TIKUS

TRHADAP EKSPRESI NESTIN DAN TRKB .

Pengenalan: Tisu kulit adalah sistem tisu yang sangat dinamik, mempunyai autoregulasi yang bertindak-balas terhadap peregangan mekanikal melalui penambahan bersih di permukaan kulit. Teknik untuk mengembang kulit dan tisu lembut banyak digunakan untuk memperbaiki permukaan tisu yang rosak dan membentuk jaringan kulit baru dengan kualiti, tekstur, dan warna yang sama. Hanya sedikit yang diketahui tentang efek pengembangan tisu kulit ini pada saraf tepi. Sekiranya saraf baru dapat dihasilkan dengan teknik ini tanpa menyebabkan kerosakan signifikan maka kulit tambahan yang dihasilkan dapat berfungsi dengan sensasi yang sama seperti kulit yang berdekatan.

Objektif: (1) Untuk membandingkan perubahan histologi antara tisu tidak berkembang dan normal dalam model tikus yang diimplan dengan pengembang tisu anisotropik. (2) Untuk menyiasat kehadiran ekspresi penanda protein nestin dan TrkB dalam tisu yang dikembangkan dan normal.

Kaedah: Dua puluh ekor tikus betina dewasa Sprague Dawley 180-220 g digunakan dalam penyelidikan ini. Sepuluh anisotropik tingkat terkontrol self-inflating tissue expansion (Oxtex, UK) diimplankan secara subkutan di daerah kulit kepala. Setelah 28 hari, haiwan di-eutanasia, ekspander dieksplan dan sampel kulit yang dikembangkan kemudiannya diambil

untuk dianalisa. Imunohistokimia dilakukan untuk menguji keberadaan ekspresi nestin (Rat 401) Mouse mAb) (CST) dan TrkB (NT-3) (CST).

Keputusan: Dua penanda protein dianalisa, nestin ditemukan di dalam sampel tisu kulit yang dikembang, keberadaan saraf setelah ekspansi kulit, sementara ekspresi TrkB sebanding dengan kulit normal.

Penutup: Analisa protein Nestin menunjukkan perluasan jaringan tisu kulit menggunakan *anisotropic self-inflating tissue expander* menghasilkan perluasan jaringan kulit dengan histologi dan kebarangkalian persarafan kulit yang hamper sama dengan tisu kontrol.

Kata kunci: saraf periferal, Nestin, TrkB, pengembang tisu anisotropik, epidermis, dermis.

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LIST OF SYMBOLS AND ABBREVIATIONS

Nestin : Neuroectodermal stem cell marker

TrkB	:	Tropomysin receptor kinase B
cm	:	centimeter
cc	:	cubic centimeters
Aam	:	acrylamide
AAc	:	acrylic acid
NIPAAm	:	N- Isopropylacrylamide
mm	:	millimeter
GBR	:	guided bone regeneration
3D	:	three dimensional
CT	:	Computerized Tomography
AP	:	arrector pili
Ig-E	:	Immunoglobulin-E
IF	:	Intermediate Filaments
CNS	:	Central Nervous System
PNS	:	Peripheral Nervous System
CDC-2-K	:	Cyclin depent-2-Kinase
G_2	:	Gap-2
M	:	Mitosis phase
DRG	:	Dorsal root ganglia
NPSC	:	Neural Stem/ Progenitor cell
mRNA	:	messenger Ribonucleic acid
FGAP	:	Fibrillary glial acidic protein
SVZ	:	subventricular zone
DG	:	Dentate Gyrus
NT	:	Neurotrophin

NGF	:	Nerve Growth Factor
BDNF	:	Brain-Derived Neurotrophic factor
NT-4	:	Neurotrophin -4
NT-5	:	Neurotrophin-5
Trk	:	Tyrosine kinase
$p75^{NTR}$	:	p75- Neurotrophin receptor
TNF	:	Tumor Necrosis Factor
kD	:	kilo-Dalton
TrkB	:	Tyrosine kinase receptor B
TrkA	:	Tyrosine Kinase receptor A
MAP	:	Mitogen Activated Protein
PI-3	:	Phosphatidylinositol-3
MAPK	:	Mitogen activated Protein Kinase
Erk1	:	Extracellular signal- regulated protein kinase 1
PI-3-K	:	Phosphatidylinositol-3-Kinase
PLC-γ	:	Phospholipase-C-gamma
SNT	:	Suc-associated neurotrophic factor- induced tyrosine-phosphorylated target
HMC-1	:	Human Mast cell line-1
IL-1β	:	Interleukin- 1β
TNF-α	:	Tumor Necrosis Factor-α
IFN-γ	:	Interferon-γ
IL-6	:	Interleukin-6
MHC	:	Major Histocompatibility complex
UV	:	Ultraviolet

°C	:	Degree Celcius
g	:	gram
SD	:	Sprague Dawley
μm	:	micrometer
FFPE	:	Formalin Fixed Paraffin Embedded.
H&E	:	Haematoxylin & Eosin
IHC	:	Immunohistochemistry
CO_2	:	Carbon Dioxide

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CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

Many surgical reconstructive procedures have been developed for the past three decades, and one of the greatest innovations is tissue expansion. This technique offers a unique potential to maintain function and form of the original tissue. Ideally, the innovation of soft tissue expansion was based on the biological properties of different tissues following application of mechanical forces that produce true tissue growth. Tissue expansion is a dynamic process and a method of gradually stretching the available (usually local tissue) tissues to generate a new and extra surface area for the availability of flap closure or transfer (Swan et al., 2012). Hence, it is a great achievement in plastic surgery which offered a solution to overcome most common problems faced during reconstructive surgery mainly due to lack of soft tissue availability.

Tissue expansion involves several phases: including preoperative planning, placing expander, removal of the expander and utilization of the expanded skin (Marcus, Horan, & Robinson, 1990). The selection of the location for placement of the expander is based on proximity to the potential defect, the area with the most laxity of skin to be easily recruited, and the best match of texture, color, and hair-bearing qualities of the skin. In general, implants are available in various shape and size but the implant chosen mainly depends on the size and shape of the defect area (Brobmann & Huber, 1985; Swan et al., 2012) (Figure 1.1 and Figure 1.2).

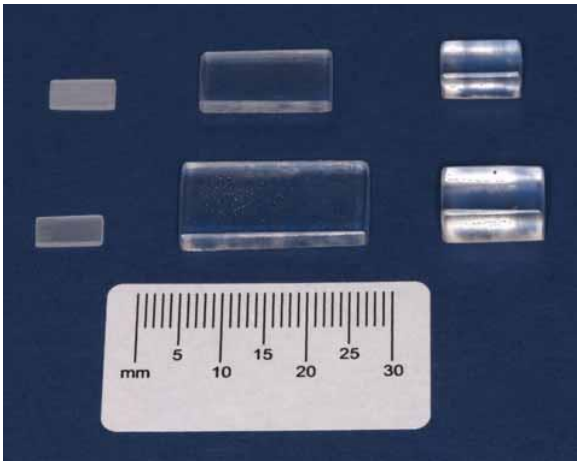

Figure 1.1: Macroscopic appearance of hydrogels expander (Swan et al., 2012). The unexpended xerogel subunits, the fully hydrated isotropic hydrogens (center), and the anisotropic hydrogels (right) are shown. Note that the direction of maximal swelling in the anisotropic hydrogels is vertical, not horizontal.

Figure 1.2: Fabricated tissue expander devices: uncoated (above) and coated (below) (Swan et al., 2012). Once fabricated, the devices were sterilized with gamma rays (Swann Morton Services, Sheffield, United Kingdom).

Basically tissue expansion yield extra tissue by exploiting the adaptability, quality and induce controlled skin growth. Tissue expansion involves a combination of creep and biological stretch. In 'creep', when a stable force is applied to the stretch skin, it progressively extend over a period of time. In "Biological stretch", the skin or any other tissue increase in surface area whenever the force is applied. In tissue expansion, the tissue is stretched without affecting the quality of original tissue. Should the force is excessive or abrupt then the dermis will rupture, resulting in striae formation. Argenta (1984), observed the softening of the overlying skin within 24-48 h of injection into a tissue expander that indicates the role of "creep" in expansion.

Following application of tissue expander on the skin, the epidermal thickness increases but after removal of the expander, the epidermal thickness gradually returns to normal after 4-6 weeks. The pilosebaceous elements are well preserved, although they may be compressed based on histological examination (Wollina et al., 1992). Hyperpigmentation is noticeable because of hyperactivity of melanocytes during expansion, however it returns back to normal slowly after removal of the expander (Pasyk, Argenta, & Hassett, 1988).

Upon histological examination, the maximum pressure effect of tissue expansion is seen in the dermis. During expansion, the dermis becomes thinner and a dense fibrous capsule is formed around the expander that continuously increase until the expander remains *in vivo*. This is a result of the presence of foreign body under the skin envelope. Contractile myofibroblasts have been seen in deep dermis near to the capsule and within the capsule. It is hypothesized that the contraction of the expander capsule is akin to wound contraction (Pasyk, Argenta, & Hassett, 1988). The magnitude of capsular contracture decreases over a duration of time because of the

intraluminal pressure in the expander. The risk of contraction increases in case of infection or exposure of the implant (Coleman, Sharpe, Naylor, Chander, & Cross, 1993). There is an association of increased metabolic activity, increased collagen synthesis and the elastin fibers become altered and fragmented (Shan & Baker, 1991). Mitotic activity of the fibroblast is maximum during the early period and, later on, it decreases. After removal of the expander, the dermis thickness returns back to normal and the capsule slowly disappears. Tissue expansion of hair bearing scalp shortens the telogen phase, possibly because of active epidermal mitosis (Lee, Gil, & Hong, 2000).

When the expander is placed over a muscle, a depression formed following implant removal and labelled as "bath-tub" depression (Figure 1.3) due to temporary compression of the muscle and not the effect of muscle atrophy (Radovan, 1984). Gur and collogues in 1998 demonstrated muscle undergoes significant atrophic changes because of long-standing pressure of the expander and lead to focal muscle fiber degeneration with glycogen deposits. When the expander is removed, the histological architecture of the muscle, vasculature and its functions return to normal.

The expansion of skeletal muscle preserves the histology of the muscle with average number of sarcomeres in the muscle fibers increases significantly. The vessels become longer with the development of the arterial network and these findings suggest an increase in the muscle length following tissue expansion as a result of biological growth process (Kim, Hong, & Futrell, 1993). It has been observed that adipose tissue undergoes permanent atrophy to the extent of 30-40% with loss of fat cells due to pressure produced by the tissue expander (Zhou et al., 2011).

The effect of expansion has been studied in experimental model, where the expander causes thinning of the cranial bone due to direct pressure but the bone density remains unaffected (Moelleken, Mathes, Cann, Simmons, & Ghafoori, 1990). Swan and co-worker in 2012 reported a 'bath-tub' depression on porcine hard palate following post-mortem in one of the porcine sample (figure 1.3). A periosteal inflammatory reaction is observed in the margin of the expander. Clinically, the cranial bones are deformed more than the long bones as the effect of the expanders, but after removal of the expander the bone deformities returned back to normal.

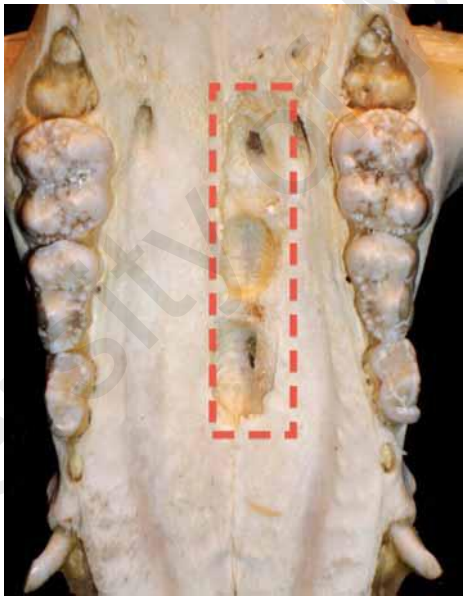

Figure 1.3: Bony palatal view of pig (Swan et al., 2012): "bath-tub" depression highlighted. Visible perforation of the palatal shelf is a result of postmortem processing of the hard palate.

The expanded skin is heavily vascularized than normal adjacent skin, where experimentally and clinically has been observed (Cherry, Austad, Pasyk, Mcclatchey, & Rohrich, 1983). Temporary hypoxia caused by the pressure of the expander is one of the explanations for the increase in vascularity or angiogenesis. A significantly higher number of vascular endothelial growth factor present in the expanded skin compared to unexpended adjoining tissue and this explained the biological effect of expanded tissue that causing angiogenesis (Lantieri et al., 1998). Generally, tissue expansion results in increase thickness of the epidermis, thinning the dermis, formation of a capsule around the expander, causes angiogenesis to improve skin vascularity and possibility of bone deformation. It was highlighted that, mechanical stretching as well as biological growth occurs during tissue expansion. On average the tissue histology will return to its original structure in a few weeks following the removal of expander. Finally, tissue enlargement is used for resurfacing the defect (Argenta & Vanderkolk, 1987).

Even though it has been used with increasing frequency to expand the size of skin and soft tissue but very little is known regarding the effects on nerves upon tissue expansion especially regarding neurogenesis and its mechanism. Recently, investigators have studied the effects of tissue expansion on peripheral nerves and the application of this technique in the repair of nerve defects secondary to traumatic injury or tumor (Kroeber, Diao, Hida, & Liebenberg, 2001).

The nerve injuries are classified based on continuity of axons and the surrounding connective tissue (Seddon, 1942; Sunderland, 1951) (Figure 1.4 and Table 1.1), and recovery from injuries related to its severity. Incomplete injuries, especially when axons retain some continuity have chances of healing. However, complete nerve injuries presented with problems; axons must re-extend through the injury site and also the progressively degenerating distal stump before reconnecting with their targeted muscle or end organ (Jiang et al, 2017). Moreover, nerves retracted after transection, adding to the size of the gap to be bridged and disrupting alignment of fascicles and endoneurial compartments. Recovery from injuries usually incomplete, particularly for proximal lesions that require extensive neuronal outgrowth, or for chronically injured nerves, when the distal stumps provide a poor regenerative environment (Mason & Philips, 2011). As a result, individual experience persistent impairment of motor function, chronic pain, and inappropriate autonomic response.

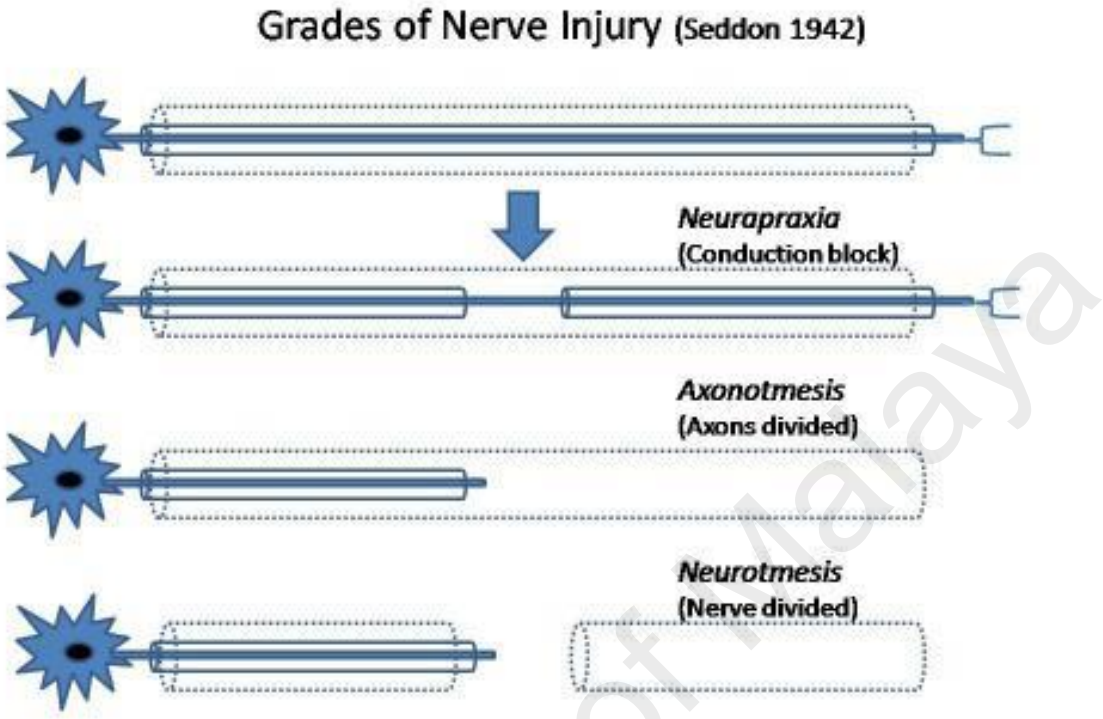

Figure 1.4: Diagram outlining the grades of injury described in Seddon's Classification (Seddon, 1942).

Seddon Group	Axon	Endoneurial Tube	Peineurium	Epineurium	Nerve conduction distal to injury	Fibrillations potentials on EMG	Sunderland Grade
Neurapraxia	+	+	+	+	Present	Absent	1st degree
Axonotemesis	-	+	+	+	Absent	Present	2nd degree
	-	-	+	+	Absent	Present	3rd degree
Neurotmesis	-	=	-	+	Absent	Present	4th degree
Neurotmesis	-	=	-	-	Absent	Present	5th degree

Table 1.1: Anatomical structures affected in each grade nerve injury of Seddon's and Sunderland's classification. ('+' = intact, '-'= disrupted) (Sunderland, 1951).

Typically, short gaps may be directly repaired end to end, while longer gaps require the interposition of a graft or scaffold between the stumps. Autografts have proven to be the gold standard in nerve repair, but associated with multiple challenges, especially donor site morbidity, limited graft supply, increased duration under anesthesia, and geometric mismatch between donor and recipient nerve sites (Schmidt & Leach, 2003). Other alternatives including guidance channels and allografts have been used, the also developed problems including immunogenicity, and for longer gaps, have yet to totally succeed (Jiang et al., 2017; Khuong & Midha, 2013).

Recent evidence suggests that moderate levels of tension may promote neural growth (Kroeber, Diao, Hida, & Liebenberg, 2001). Nerves are not a prototypical load-bearing tissue, but they do exist in a dynamic biomechanical state. They are exposed to loading during joint movement and development (Topp & Boyd, 2006), in some cases experiencing regional strains approaching 30 % (Wright, Glowczewski, Cowin, & Wheeler, 2001). When the deflection exceed levels that induce injury (Brown et al., 1993), and likely reflect regional differences in nerve structure and biomechanical properties to adapt increased strain (Mason & Philips, 2011).

This regional heterogeneity may also reflect a nerve's ability to meet its functional needs by adapting to its mechanical environment (Vaz et al., 2014). Recent work at the cell and tissue scales has revealed the importance of tension as a growth modulating signal and possibility of a survival signal (Anava et al., 2009). Under physiologic loads, tension induces growth. Clinically, empirical evidence from the use of limb lengthening procedures in orthopedic surgery and tissue

expanders in plastic surgery has demonstrated that nerve strain is often a rate-limiting factor, but at proper strain rates, large amounts of limb growth or tissue expansion can be produced without impaired nerve function (Stanitski, 1999).

Individual neurons in cell culture response well to tension. Reports revealed that towing axonal growth cones promotes axonal elongation (Zheng et al., 1991). Neurons reached lengths exceeding 10 cm, at a maximal rate of 8 mm per day of regeneration and maintained their diameters and executed cellular processes such as axonal transport at pre-strain level (Loverder et al., 2011), demonstrating an active response to deformation.

Elongation of intact proximal stumps towards the injured distal stumps of severed peripheral nerves can reproduce the mechanical environment created during *in vivo* and *in vitro* procedures. Lengthening across a nerve gap may accelerate functional recovery and ensures an anatomic and functional match to the distal stump. Reports from previous study revealed that the use of a modified external fixator to close nerve gaps in rats, rabbits and monkeys (Vaz et al., 2014); Yamada et al., 2009) (Figure 1.5), where this device exerted tension on both the proximal and distal stumps of the nerve. After the gap was closed, direct end-to-end repair was performed following device removal. It is possible that extracellular matrix and compacted lamina in the distal stump are actively remodeling in response to strain. As a result, imposed strains are well beyond structural tolerances for nerve loading (Georgeu et al., 2005), and regenerating axons would have to extend a longer distance through a mechanically compromised environment to reach targets.

Intact rat sciatic nerves, were rapidly elongated up to 24% through the use of an underlying tissue expander (Arnaoutoglou et al., 2006), but resulted in partially reversible nerve conduction deficits and histological changes, including myelin thinning. The conductive changes following the use of a tissue expander may be attributed to widened nodes of Ranvier (van der Wey et al., 1996), with blood flow preserved for expansion up to 40%. On the other hand, changes in conduction and nerve morphology are indicative of a compression neuropathy, and possibly, a related tension neuropraxia due to the relatively short time over which this devices act.

In the recent years, nerve lengthening using tissue expander and longitudinal lengthening of the nerve stump using external nerve distraction device has been studied but none focused on neurogenesis following tissue expansion (Vaz et al., 2014). Hence, this aimed to investigate the morphologic and cellular response of the peripheral nerve by tissue expander using animal model. This study also aimed to detect the influence of mechanical tissue expansion on neurogenesis and expression of nestin and TrkB in expanded skin.

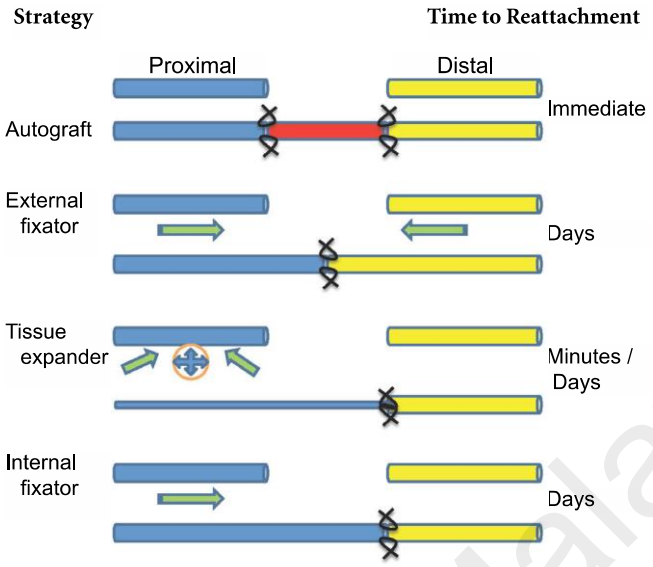

Figure 1.5: Schematics of nerve lengthening strategies (Vaz et al., 2014)

Blue: Proximal stump; yellow: distal stump; red: autograft (or other graft).

Arrows represent direction of tension placed on respective stumps.

Sutures are indicated at stump-graft or stump-stump interfaces.

1.2 Importance of Proposed Research

Although contemporary surgical techniques provides acceptable closure of the majority of cleft palate defects, a small but significant proportion of severely hypoplastic, wide or previously scarred clefts present with formidable problems. The frequent use of lateral releasing incisions to facilitate tension- free closure delivers added pain, scarring, and prolonged time to wound healing. Reconstructive procedures utilized by plastic surgeons often benefit from extra epithelial and supportive tissue that can be obtained through tissue expansion as a technique providing an innervated flap with minimal donor site defect. The ability to use local tissues depends on the nature of the defect and the availability of adjacent tissue for local advancement. In the absence of local tissue for reconstruction, other options such as regional or free tissue transfers are use. However, sensory testing on patients with tissue expanders in situ and after flap replacement has given variable results, indicating that sensation can be affected. The mechanism of altered sensation may be surgical or due to the force of expansion. The pressure of expansion in the acute phase may affect nerve conduction, which would be expected to return to normal as the pressure falls. In the long term, sensation is altered by surgical dissection as well as by the mechanical forces in the tissue. The skin responds to the forces of expansion by recruitment, stretch, and increased mitosis. The pathophysiological changes with regards to the vascular and support elements are well documented but detailed regarding neurogenesis has not been investigated. Hence, this study was designed to investigate the neurogenesis of peripheral nerve following application of mechanical tissue expansion which possibly useful in flap transfer with similar innervations as adjacent tissue. With this technique, it could eliminates the need for nerve graft that have to cross two anastomoses and regeneration of nerves occurred outside the nerve

fascicles. Moreover, it helps to eliminate donor site defects following extraction of nerve graft from distance donor site.

1.3 Aim

This study aims to detect the influence of anisotropic tissue expander in cranio-facial region

1.4 Objectives

i. To distinguish the skin morphology between tissues implanted with the expander and control tissue.
ii. To evaluate and compare the histological changes caused by anisotropic tissue expander between study and control group.
iii. To evaluate the presence of nestin and TrkB protein marker expression between expanded and non-expanded skin.

1.5 Null Hypothesis

i. There are no difference in terms of skin morphology in tissue implanted with tissue expander and tissue without tissue expander.
ii. There are histological changes in tissue implanted with tissue expander and tissue without tissue expander.
iii. There are no difference in regards to nestin and TrkB expression between two groups.

CHAPTER 2: LITERATURE REVIEW

2.1 Tissue Expander

2.1.a History of tissue expander

The first few attempts on "tissue" expansion were directed not at soft-tissue model, but focused on bone elongation. Codivilla (1905), reported procedural of femoral elongation using external bony traction followed by Magnuson (1913) who used an external traction device and surgically lengthen shortened bones in the leg. They also reported that this could be used to successfully and concomitantly stretch the soft-tissue of the leg. After that, Putti (1921) from Italy, showed that sustained traction in the bone over a duration of time could result in 8-10 cm of lengthening not only in the bone, but also in the vital soft-tissue structures such as the muscles, nerves, and blood vessels, as a confirmation to the Illizarov method (Ilizarov, 1988).

The history of soft tissue expander were first developed by Dr. Neumann (1957) from New York, who applied a subcutaneous rubber balloon to expand skin tissues in order to repair an ear defect. Two decades later, Radovan in 1976 and 1978 reported a technique of skin expansion that used a silicone expander inflated by percutaneous injection of saline through a remote injection port. (Austad & Rose, 1982) reported their findings on an implant that used a sodium chloride gradient to achieved gradual tissue expansion without injection (Figure 2.1).

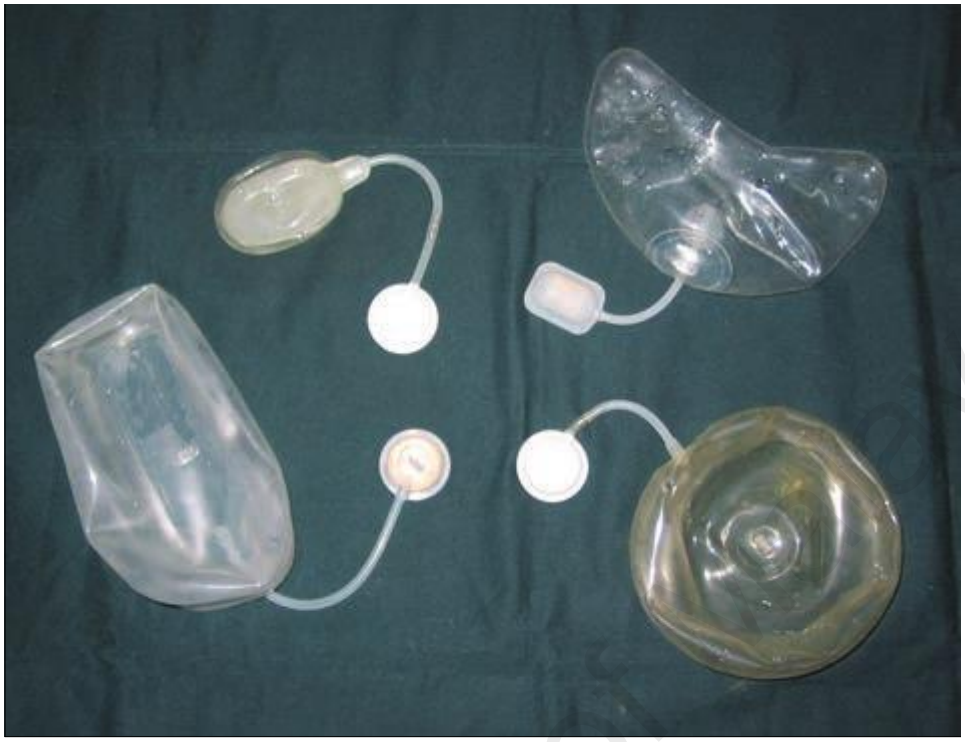

Figure 2.1: Different types of tissue expanders (Wagh, 2013).

Meanwhile, with the conventional expanders, the amount of soft tissue generation has been predetermined based on the type of expanded tissues and the shape of the expanders (Brobmann & Huber, 1985; van Rappard et al., 1988). It was observed that more tissue gain with rectangular and crescentic forms compared with round-based expanders (Johnson et al., 1993). Despite the positive feedbacks with the conventional expanders, they have several disadvantages, such as repetitive inflations, which may increase the treatment duration up to several months. The intermittent modality of external inflations creates pressure peaks with a reduction in the tissue vascularity (Pietila, 1990), and may cause an expander perforation through the soft tissue (Wiese, 1993). A lack of perfusion caused by increased in pressure reduces the local oxygen partial pressure of the soft tissues and therefore increases the risk for expansion failures (Berge et al., 2001).

In addition, serial injections increase the costs of treatment and morbidity of the patients as well as the risks for adverse effects by repeated punctures. Despite these drawbacks, conventional soft tissue expanders are still used in plastic surgical procedures. As a result of the above mentioned shortcomings, the use of conventional expanders is limited in cranio-facial defects (Van Damme et al., 1992). To overcome the disadvantages of conventional soft tissue expanders, Austad and Rose (1982) developed a self-inflating osmotic soft tissue expander, without an external port and the need for repetitive inflations. This new type of expander was based on a semi-permeable silicone membrane which contained hypertonic sodium chloride solution. With the principle of osmotic gradient, it allowed a continuous inflow of body fluids into the expander. As a consequences, the expander volume increased with concomitant soft tissue growth. The Austad expander suffered from technical problems including, long inflation

times compared to Radovan type of expander and the potential for exposure of surrounding tissues to hypertonic saline in the event of rupture including possibilities of tissue necrosis (Austad & Rose, 1982). The Austad expander require only 8 to 14 weeks, while Radovan expander only need 6 week or less to give its effect. Thus Radovan device has become the dominant tissue expansion implant in the current use. The expanders used in these early stages were made of silicone rubber, with an external valve penetrating the skin for manual inflation by serial injections (Radovan, 1984).

In 1993, Wiese developed a novel self-inflating osmotically active soft tissue expander made of hydrogel in order to overcome major drawback from Austad and Radovan-type of expanders. It consists of both a polymer network (cross-linked hydrogel of co-polymers based on methyl-methacrylate and N-vinyl-pyrolidone) including variable aqueous component. Since 1999, this hydrogel expander has been designed and manufactures under the name of Osmed® (Ilmenau, Germany), which is the first commercially available self-inflatable osmot1c expander and has been FDA- approved since 2001 (Wiese et al., 1999).

The biomaterials used are the same like in contact lenses and offer a higher biocompatibility without eliciting any toxic effects, adverse immune reactions, infections or any other systemic manifestations, and most importantly, they do not produced any localized inflammatory reactions in the soft tissues (Wiese et al., 2001). In general, the incorporation of methacrylate, produces ionic hydrogels due to the presence of carboxyl moieties, which results in greater osmotic potential and subsequent amplification of the swelling capability in comparison with non-ionic hydrogels. The inclusion of “methyl-methacrylate”, specifically, in osmotic

hydrogel expanders results in increased of swelling ratio (Wiese, 1993; Wiese et al., 1999) compared to the presence of "hydroxyethyl-methacrylate" (Mazzoli et al., 2004).

The presence of cross-links renders the polymer network insoluble in aqueous media (Bell & Peppas, 1996), therefore, the expander has the ability to enlarge and retain extended volumes produced by swelling and not liquefy in the aqueous media. In an attempt to test different biomaterials, Varga et al. (2009) developed a hydrogel osmotic soft tissue expander made of either acrylamide (AAm), acrylic acid (AAc) or N-isopropylacrylamide (NIPAAm). Although NIPAAm hydrogels were proven to be the most appropriate biologically and mechanically for applications in plastic and reconstructive surgeries, these expanders were only tested *in vivo* and remained to be validated in clinical trials.

An osmotic expanders terminates the need for multiple injections, inflate continuously by osmotic gradients without the need for additional interventions. A constant expansion compare to an intermittent inflation results in the formation of new cells, tissue growth (van Rappard et al., 1988), and a greater amount of final tissue gain (Bascom, 2002; Bennett & Hirt, 1993; Wee, Logan, & Mustoe, 1992).

Absence of an external filling port minimize the bulkiness of the expansion device (Swan et al., 2012), which facilitates the positioning of the expanders. Osmotic expanders are initially smaller in size compared to conventional expanders with starting volume of 10% from its final volume (Ronert, Hofheinz, Manassa, Asgarouladi, & Olbrisch, 2004). Following that, osmotic expanders will only require a smaller incision for placement (Chummun, Addison, & Stewart, 2010), which helps reducing the surgical trauma. Miniaturized osmotic expanders have been successfully used in clinical ophthalmology (Schnittkowski, Knaape, Gundlach, Fichter, & Guthoff, 2003) and exposed a new pathways in pediatric surgery (Obdeijn, Nicolai, & Werker, 2009).

There are two generations of Osmed® hydrogel soft tissue expanders (Figure 2.2). The first generation lacks of silicone envelope surrounding the surface of the hydrogel which results in extremely rapid expansion in the early stages after insertion with subsequent side-effects (Rees, Morris, & Hall, 2008). Importantly, rapid inflation does not result in an actual increase in soft tissue volume because there is chances that tissues might return to their pre-expansion state following rapid expansion (Johnson et al., 1993).

To overcome the undesirable outcomes, a second generation of osmotic hydrogel soft tissue expanders, coated with silicone, was introduced in 2001 (Anwander et al., 2007; Ronert et al., 2004). On the other hands, the inclusion of silicone coating adjusts the expansion speed overall, which gives ample time for the newly formed tissues to adapt, more time for wound healing and greater amount of expanded tissue (Wee et al., 1992), including effective soft tissue regeneration (Wiese, 1993; Wiese et al., 2000).

Figure 2.2: Osmed® hydrogel soft tissue expanders (Lohana, Moiemen, & Wilson, 2012).

2.2 Application of tissue expander in head and neck region

2.2.1 Scalp

Functional and aesthetic subtleties make reconstruction of the head and neck extremely difficult. Problems with inadequate local tissues or large defects, resulting in obtaining flaps from a distance for reconstruction. Distant flaps may provide bulk for symmetry and function, but the aesthetic quality leaves consequence. The use of expanding prosthesis allows local tissue of the face to be augmented for the use in reconstruction. The skin expansion process offers quantity of tissue and quality, since "donor site" identical to the recipient site. The donor site remains covered with portions of the expanded tissue, avoiding secondary reconstruction (Argenta, Watanabe, & Grabb, 1983).

Currently, the trends on using the technique of tissue expansion in the head, face and neck region are increasing. Multiple surgical technique seems to have been developed and based more on clinical experiences and less focused on experimental research. The main purpose of tissue expansion is to create tissue with normal texture, colour, thickness, with preservation of sensibility, in order to cover a certain defect without creating a donor site defect. In cranio-maxillo-facial surgery, the following specific reasons for tissue expansion can be identified: firstly, to increase the amount of specific soft-tissue including scalp, forehead, periorbital tissue, nose, face and neck. Secondly, to improve the blood supply to a particular region with decreased vascularity, with the aim of improving the viability and long term stability of bone or cartilaginous grafts (Argenta & Vanderkolk, 1987).

Tissue expansion involving the scalp started since 1982, with numerous authors published successful clinical reports on correction of baldness and traumatic defects (Argenta, 1984; Radovan, 1984). Several case reports published the successful scalp reconstruction in aplasia cutis congenita and separation of craniopagus twins (Argenta & Dingman, 1985; Snyder, 1985), and reports on microvascular transplantation of expanded free scalp flaps between identical twins (Valauri, Buncke, Alpert, Lineaweaver, & Argenta, 1990)).

Utilization of tissue expanders for treatment of post-burn contractures and post-burn alopecia has been reported (Buhrer, Huang, Yee, & Blackwell, 1988; Chang & Jin, 1986; Cooper & Brown, 1990; Kasai, Ogawa, & Takeuchi, 1991; Leonard & Small, 1986; Neligan & Peters, 1989) and it was adjudged as valuable, simple, safe and reliable technique that allows reconstruction of defects (Van Damme et al.,1992).

2.2.2 Cheek and Neck

The face is highly complicated part in human body, as such restoration of form and function following injury or disease of the face is important to the psychosocial health. It is impossible to recreate nature, the goal of the reconstructive surgeon to create the illusion of normal as due to demands to meet this goal vary with the location of the facial soft tissue defect. The face may be divided into central and peripheral unit (Menick, 1987) (Figure 2.2). The cheek and forehead is the peripheral unit, in addition to its role in facial expression, it provides the architecture for mastication, deglutition and speech. The cheek borders consist of eye, nose, lips, chin, mandible, and ear. In the buccal region, the cross-sectional anatomy medially outwards includes the buccal mucosa, the submucosa, the buccinator muscle, the buccal space, the deep

fibroadipose connective tissue layer, the superficial musculoaponeurotic system, the superficial fibroadipose connective tissue layer, dermis and epidermis.

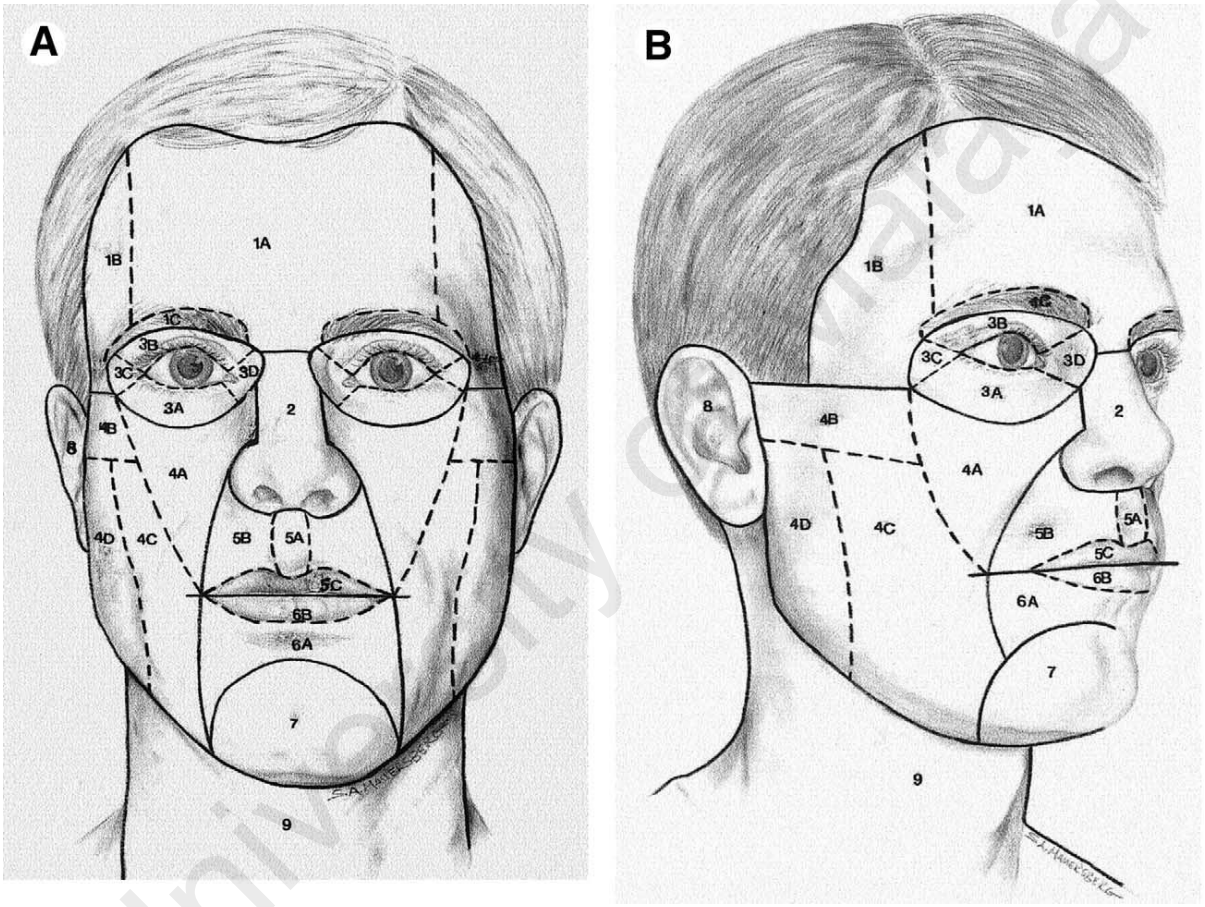

Figure 2.2: Facial aesthetic units (Menick, 1987).

Central facial units include the eyelid, nose, lip and the subunits are alar of the nose, upper lateral eyelid, and lower lateral lip (Figure 2.2). They have complex and subtle contours and any asymmetrical with the contralateral subunit is immediately obvious to the observer. When reconstructing the central subunits, it is essential to match skin contours and outlines together with skin color and texture. In contrast, the outline of the contralateral cheek cannot be easily compared for symmetry purpose. It is often aesthetically sufficient to match skin color and texture with minimal contour and outline. The facial reconstructive surgeon is armed with a plethora techniques tailored for different cheek defects (Menick, 1987).

The cheek and neck can be considered a single anatomic location form the standpoint of tissue expansion due to its skin vascularity, texture and thickness are similar. Expansion of cheek and neck skin is primarily indicated in reconstruction of defects involving neck or lower third of the face in situations where local cutaneous flaps would be inadequate in size or vascularity without the benefit of expansion. Expansion resulted an increase in the amount and vascularity of suitable donor skin for restoration of large face and neck defects. The enhanced skin can be mobilized as a random advancement or transposition flap with a high length-to width ratio (Spence, 1988). The enhanced skin also offered other great advantage where the skin have similar texture and color to the defect region.

The expander is placed subcutaneously in the cheek and deep to the platysma muscle in the neck through a small incision localized at the area to be expanded. The incision is carefully designed so it will not interfere with incisions for subsequent reconstruction. It is recommended to place the expander over rigid foundation to maximize the vector forces of expansion outward

toward the overlying skin. Thus, the base of the expander should be centered over the bone of the malar eminence or the mandible in the cheek and over the transverse processes of the cervical spine in the neck.

Gravitational forces are more influential in migrations of expanders placed in the cheek and neck than in the forehead and scalp. This will prevent inferior migrations, it is recommended to secure the base of the expander to underlying tissues using a permanent sutures (Baker & Swanson, 1990). Tissue expansion progress by weekly injection of saline. Following optimum expansion, definitive reconstruction is initiated by removing the expander through incisions to provide appropriate cutaneous flap for reconstruction. It may necessary to score the fibrous capsule surrounding the expander to permit optimal mobilization and draping of the expanded skin, but removal of the capsule is discouraged. Suction drainage at the time of reconstruction is usually necessary because of the enhanced vascularity to the expanded skin.

Tissue expansion of the cheek and neck is distinguished by a particularly high rate of complications. The most common is exposure of the balloon or injection port of the expander. Antonyshyn and co-workers in 1988 reported 69% incidence of complications in 14 patients undergoing cheek and neck skin expansion with pooling of injected saline in the most dependent portion of the expander and subsequent gravitational descent of the implant. Further inferior displacement was prevented by anatomical structures, such as lower margin of the mandible or the clavicle, the expander tend to fold and buckle, causing progressive erosion of the skin directly overlying these folds and subsequent exposure of the implant. The dependent pooling of

injected saline causing disproportionate expansion of tissue occurs over the inferior aspect of the expander.

Greater than 50% of the expander extruded when used for skin expansion of the cheek and neck is related to several factors, especially in relative thin skin and subcutaneous tissue and the lack of adequate bony foundation upon which the implant rest. With a constant shearing forces resulting from motion of the skin on the face and the pronounced gravitational influence are other important facts (Antonyshyn et al., 1988).

2.2.3 Auricular

Neumann (1957) reported the utilization of tissue expander to obtain cutaneous cover for cartilaginous graft in case of subtotal reconstruction of the ear following partial traumatic amputation. According to Neumann, a rubber balloon was gradually distended by successive insufflation with air. Following that, several other case reports have been published regarding auricular reconstruction with the aid of soft-tissue expansion (Chana, Grobbelaar, & Gault, 1997; Liu, Sun, & Li, 2011; Oneal, Rohrich, & Izenberg, 1984; Quaba, 1988; Sasaki, 1989; Tanino & Miyasaka, 1990) (Figure 2.3).

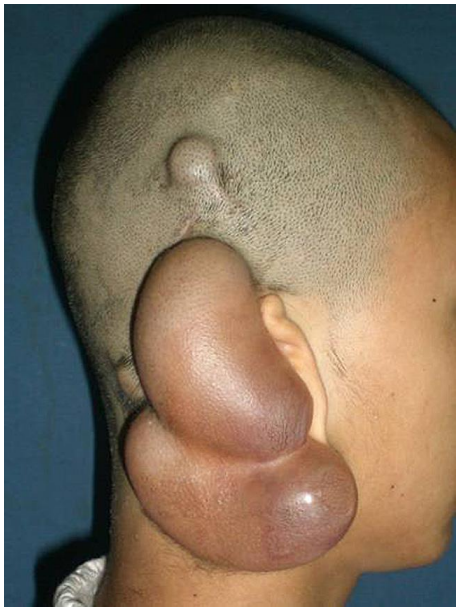

Figure 2.3: Two skin expanders implanted at the auricular region (Liu, Sun, & Li, 2011).

2.2.4 Eyelid and orbit

There is limited experience with tissue expansion in oculoplastic surgery (Víctor & Hurwitz, 1984; Víctor, Hurwitz, & Gruss, 1986). Its utilization primarily in the surgical management of cicatricial ectropion of the lower eyelid, although tissue expansion has application in reconstruction of large eyelid defects so that defect can be closed without free grafts. A cigar-shaped, 1.2-cc volume silicone expander measuring 10 x 30 mm is used (Antonyshyn et al., 1988). Tissue expander is placed postero-laterally in the temporal region. The base of the expander will be positioned inferior to the inferior orbital rim to prevent compression of the ophthalmic globe. Skin expansion of the eyelid is associated with a risk of implant extrusion, and it has been reported that two out of six patients undergoing eyelid expansion suffered exposure of the implant and required immediate removal (Antonyshyn et al., 1988). The possible role of soft tissue expansion in anophthalmic orbits has been rule out by (Berry, 1991; Lo et al., 1990).

2.2.5 Forehead and nose

Expanded forehead skin is used primarily for repair of forehead defects or as a preliminary step in nasal reconstruction. Various lesions affecting the forehead can be removed and primary closure of the wound achieved when other sites of the forehead was expanded. In treating forehead deformities, expansion simultaneously expands skin and frontalis muscle, generating a sensate musculocutaneous flap consisting of innervated, striated muscle for restoration of forehead expression. Placement of the expander through a hair-bearing scalp incision permits maintenance of a natural hairline (Adamson, 1988).

Forehead expansion is beneficial when forehead flap is required for nasal reconstruction and there is insufficient tissue to allow for both reconstruction of the nose and primary closure of the forehead donor site. Tissue expansion can provide ample skin for total nasal reconstruction with a midline forehead flap, thus obviating the need for deforming scalp or total forehead flaps. Flaps generation as wide as 6 cm to 7 cm and allowing primary wound closure. Patient with low hairline, it is possible to generate a flap with sufficient vertical length excluding the hair-bearing scalp (Baker & Swanson, 1990).

Although the techniques on forehead expansion are similar to the scalp, but expansion on forehead skin is more painful due to stretching of the supratrochlear and supraorbital nerves but manageable via oral analgesics. Generally, durations to achieve sufficient tissue for total nasal reconstruction are around 2 months using weekly inflations. Primary closure of the forehead results in an acceptable single vertical midline scar. Lateral extensions of the flap are needed to

achieve sufficient tissue in alar reconstruction. In such instances, primary closure is achieved using a T-shaped closure (Adamson, 1988).

Adamson (1988) recommended subcutaneous placement of the expander, while Baker and Swanson (1990) suggested placing the expander in a subgaleal pocket. The overlying galea and frontalis muscle appear to protect against excessive thinning of the forehead skin and causing implant extrusion. A coronal incision of 4 cm posterior to the hairline was recommended because incisions made too close to the hairline may cause in wound dehiscence and implant extrusions. A recipient pocket for the expander should extent to the supraorbital rims and sufficient to allow the base of the expander to rest against the frontal bone without folding. A 250-cc volume rectangular expander is appropriate for majority of the cases that need forehead expansion.

A preliminary step in total nasal reconstruction using forehead expansion is effective in providing a large, well-vascularized midline forehead flap. With increased vascularity of the flap it allows removal of the frontalis muscle and aggressive thinning of the subcutaneous fat at the time of transposition. At the same time, the fibrous capsule surrounding the expander in the vicinity of the distal flap should be removed. These surgical methods provide a very thin, flexible flap for contouring and restoring a delicate columella and nostril. A thinned flap can be turned on itself to reconstruct full thickness defects of the nasal alar and vestibule without producing excessive bulky nose. In the case of major full thickness defects of the nasal dorsum, internal lining can be provided by turned-down flaps from the adjacent skin to the defect. Some contraction of the expanded flap will be compensated by designing the flap to increase its length.

It is important to provide skeletal framework of the nose such as bone or cartilage grafts at the time of flap transposition of detachment (Baker & Swanson, 1990).

In cases of craniofacial deformities associated with hypertelorism, facial clefting and low hair line, tissue expanders offers solution including expansion of the forehead, correction of the hairline by placement of expander beneath the frontalis muscle in the scalp (Argenta & Vanderkolk, 1987). The success of using tissue expanders in correcting nose and covering larger defect by creating larger forehead has been reported (Adamson, 1988; Argenta & Vanderkolk, 1987; Cole, Gault, Mayou, & Davis, 1991; Kroll, 1989; Toth, Glafkides, & Wandel, 1990). The success of nose reconstruction using tissue expander directly on the skin of the nose was reported by Argenta and Vanderkolk (1987); Hirshowitz, Kaufman, and Ullman (1986); Muenker (1988b); Sasaki (1988) (Figure 2.4).

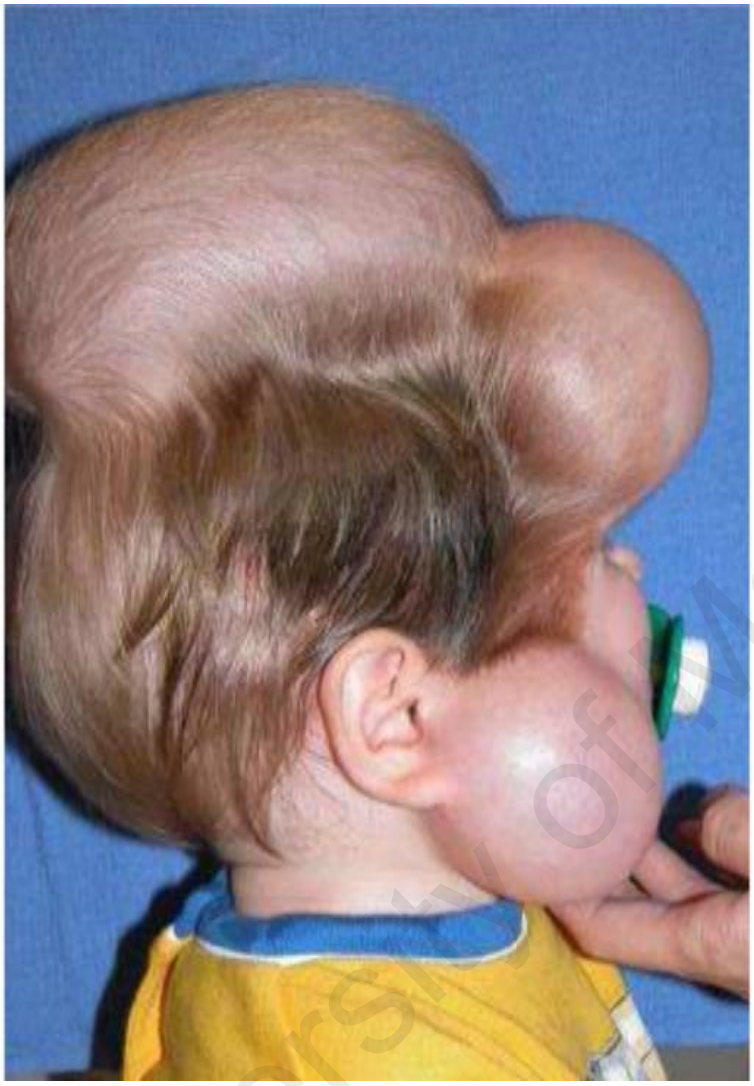

Figure 2.4: Tissue expansion for pediatric forehead reconstruction (Zöllner et al., 2012).

2.2.6 Intra-oral applications of soft tissue expanders

In intra-oral surgery, the application of expander was first described by Argenta and Vanderkolk (1987). The use of soft tissue expanders prior for bone augmentation of the severely atrophic mandibular ridge was reported by (Bahat & Handelsman, 1991; Lew, Amos, & Shroyer,

1988; Wittkampf, 1989; Zeiter, Ries, Mishkin, & Sanders, 1998). In the above cases, conventional expander in the shape of silicone balloons were applied and they included few patients without long- term follow-up, in terms of stability or relapse of expanded soft tissues and outcomes of hard tissue procedures. Recently, the application of osmotic hydrogel soft tissue expanders of the second generation in intraoral region have been investigated.

Uijlenbroek et al. (2011) tested osmotic hydrogel soft tissue expanders in the palatal mucosa of goats to validate the effectiveness and efficiency of soft tissue expanders in various intra-oral applications. As the palatal mucosa is very firm in these animals, the researchers hypothesized that a similar expansion would be successful in the oral cavity of humans. Expanders were implanted in duration of 40 days, using either a "tunnel" approach or a "flap" approach. After swelling, the expanders had created a surplus of soft tissues with an excellent shape and no signs of inflammation. Histologically revealed no signs of bone resorption, despite the pressure exerted on bone, which is equal to the amount of pressure needed to expand the soft tissues. In comparison to the technique use for expander insertion, they reported no difference was observed between the tunnel and the flap approach. With the tunnel technique, fixation of the expander was more challenging compared to flap approach due to limited view and freedom of handling the expander. Based on the manufacture's guidelines for intra-oral use of the expanders, insertion is recommended with the "tunnel" technique as this technique prevents a complete flap reflection for expander placement.

Abrahamsson, Isaksson, and Andersson (2011) placed sub-periosteal osmotic soft tissue expanders in mandibles of rabbits. In each rabbit, two sites were identified: test site in contact

with the base of the expander and control site with the flat end of the expander, fixed by a mini screw and has no expanding capacity. Two weeks post-expansion, clinical examination showed no signs of soft tissue dehiscence or infections and histological study revealed periosteal expansion without any signs of inflammatory reactions or bone resorption. In fact, new bone formation at the edges of the expanded periosteum was present, while there were no signs of bone formation in the control group.

In the following study, the authors applied the same animal model and protocol in order to evaluate the outcomes of post- expansion bone augmentation by guided bone regeneration (GBR- only bone graft and covered either by titanium mesh or bioresorbable mesh) (Abrahamsson, Isaksson, Gordh, & Andersson, 2010). Three months post-augmentation, it was evident that tissue expanders were able to create a sub-periosteal space and new bone formation was allowed underneath the mesh and at the edges of the expanded periosteum. The effect of soft tissue expansion on the outcomes of GBR with two different grafting materials was evaluated in another animal study (Abrahamsson et al., 2011) (Figure 2.5). In agreement with previous findings, soft tissue expanders were able to create a surplus of soft tissues including periosteum, in facilitating mucosal coverage of the bone graft without occurrence of soft tissue dehiscence. New bone formation was found under the titanium mesh regardless of the type of bone graft. Nonetheless, it must be noted that a lack of soft tissue dehiscence or related complications in this report may be attributed to the adopted extra-oral surgical approach. The authors chose such an approach as the access via the oral cavity in rabbits was restricted (Abrahamsson et al., 2010).

There are few clinical data available which describe the mucosal expansion prior to bone augmentation; two case series (Kaner & Friedmann, 2011; Mertens et al., 2015) (Figure 2.6) and one randomized controlled clinical trial (Abrahamsson, Wälivaara, Isaksson, & Andresson, 2012) could be found in literature.

Figure 2.5: Osmotic soft tissue expander (a) before expansion, (b) after expansion (Abrahamsson et al., 2010).

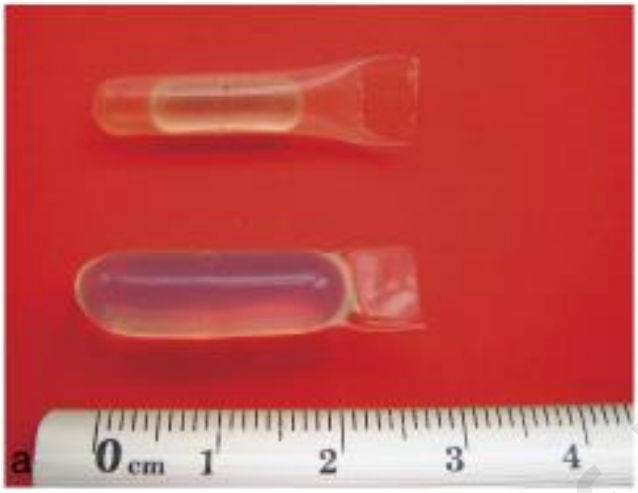

Figure 2.6: cylindrical tissue expander before and after swelling (Kaner & Friedmann, 2011).

In a randomized controlled clinical trial, Abrahamsson et al. (2012) applied sub-periosteal soft tissue expanders in ten patients requiring bone augmentation prior to implant placement. Two weeks post-insertion, the expanders have been removed and GBR was carried out with either a particulate onlay graft protected by titanium mesh and a collagen membrane (test group), or a cortical bone block graft, harvested from the ramus, without any previous soft tissue expansion control group). The authors choose GBR as the bone-grafting method in the test group because it gave predictable results as reported earlier in regards to bone fill (Degidi,

Scarano, & Piattelli, 2003) and promising results after mucosal expansion have been described in previous animal experiments (Abrahamsson et al., 2010).

In the study group, two subjects showed soft tissues perforations due to expander placement close to incision line. However, in these situations, the soft tissue expander was sufficient to completely cover the bone graft with the mucosal flap. Meanwhile, in the control group, periosteal incisions were required to allow flap advancement and achieve full coverage of the bone grafts (Abrahamsson et al., 2010).

Evaluation in changes of the soft tissue profile on the attached gingiva were done at baseline and 6 months after augmentation in both groups and additionally at post expansion in the experimental group using an objective 3D metering device. This device is based on digital light stripe projection which deflects whenever the surface alters in topography. Deflection data are registered through a sensor and stored in a computer with appropriate software together with the clinical photo captured. Data are evaluated and displayed as a color coded picture of the topography (Wälivaara, Isaksson, & Rosén, 2007). The mean soft tissue profile gain at the attached gingiva level was 2.9 $\pm$ 1.1 mm compared to baseline record, while it decreased to 2.3 $\pm$ 2.1 mm at the time of implant placement compared with the starting point. The control group showed a soft tissue profile change of 1.5 $\pm$ 1.4 mm at the time of fixture installation. Even if the experimental group showed increased gingival dimensions after surgeries, the differences were not statistically significant (Abrahamsson et al., 2010).

Six months post-operatively, the study group showed a minimal resorption of bone graft in the vertical dimension of just 27% and 14% tendency for resorption in the horizontal aspect. Corresponding to previous findings in the literature (Chiapasco, Zaniboni, & Boisco, 2006; McAllister & Haghighat, 2007), vertical bone resorption was more prominent than lateral side. On the other hand, the control group showed statistically significant bone resorption in both the vertical (42%0 and horizontal (23.5%) dimension. Overall, bone resorption in the experimental group was less pronounced that that in the control group. However, the difference just reached statistical significance when smokers have been excluded from the calculation. Smokers have been included in the study as they might be candidates for such an approach in everyday clinical practice (Abrahamson et al., 2010).

The favorable outcome with expansion could be attributed to the direct contact of the bone graft with periosteal progenitor cells. One might speculate that a reduced bone graft resorption in the test group is due to different augmentation modalities in both groups. Despite the success results reported with soft tissue expansion in animal and human clinical trials, the authors recommended further refinements of the soft tissue expansion technique particularly in smoking patients by focusing on the risk reduction in complications such as soft tissue perforation (Asa'ad et al., 2016).

In addition, positive outcomes of pre-augmentation soft tissue expansion were reported by Kaner and Friedmann (2011). In opposition to the previous study, the osmotic expanders have been placed in submucosal pouches. The reason for the altered location was to prevent replacement of periosteum with collagen-rich connective tissue lacking osteoblasts and precursor

cells, which may have negative effects on the healing of subsequent bone graft. From twelve patients enrolled in the study, two experienced soft tissue perforations by the expanders and had to be retrieved before final expansion. Perforation present due to infection after 4 weeks of insertion in one patient, while the choice of an oversized expander was the cause in the other case; a fact that emphasizes the selection of an appropriate size of the expander. Perforated sites were allowed to heal for 6 weeks and then retreated with small expanders (Kaner & Friedmann, 2011).

After 60 days post-insertion, all expanders reached their final volume and the vertical bone augmentation was carried out with onlay grafting (autogenous bone block harvested from the ileum in three patients) or GBR with ramus graft covered with Bio-Oss and a collagen membrane). At the time of expander removal, a surrounding capsule of soft tissue present and did not show signs of inflammatory infiltration upon histological analysis. The expanded tissues showed good quality, and the space created by the expanders allowed a tension free primary closure. Even though the occurrence of a minor exposure of bone graft in one patient following local debridement without any further complications. In the current study, pre-augmentation soft tissue expansion reduced the incidence of post-operative graft exposure to 4% in comparison with the previous studies of vertical bone augmentation without prior soft tissue expansion of 23% (Verhoeven, Cune, Terlou, Zoon, & de Putter, 1997), 27.3% (Chiapasco et al., 2006), 25% (Proussaefs & Lozada, 2005), and 22% to 25% (Merli, Migani, & Esposito, 2007). A cone beam computed tomography (CBCT) was analyzed after 6 months of bone graft healing and before implant placement, resulted a high vertical bone gain 7.5 $\pm$ 2.4 mm, compared to findings from a systematic review (Jensen & Terheyden, 2009) revealed mean vertical bone gain by 4.8 mm with

multiple augmentation methods. Unfortunately, the amount of bone resorption was not measured from the case series. Bone biopsies were histologically analyzed with micro- computed tomography (micro-CT), revealing appropriate bone volume density with apparent trabeculae structure.

In an alternative case series, sub-periosteal osmotic expanders were applied by tunnel approach in eight patients with severe atrophy in the maxilla or mandible before bone augmentation (Mertens et al., 2015). Quality and quantity of newly created soft tissues were evaluated together with post-operative soft tissue-related complications such as perforation, infection, dehiscence, necrosis and pain. Expansion time varied between 20, 40 and 90 days depending on the size of the defect and size of the expanders. Following insertion, patients complained a pressure at the insertion area without association any symptoms of pain. The only soft tissue- related complication was mucosal perforation and eventual pre-mature loss of expander. The complications occurred in two patients; one had a history of previous trauma at the area of implantation, and the other had a cleft surgery and failed to follow the post-surgical instructions of avoiding wearing the prosthesis. The patients showed signs of mucosal scars prior to insertion of the expander. The case selection for soft tissue expansion is important to avoid complications and presence of scars at the surgical site might be contraindicated for soft tissue expansion. Previous animal studies (van Damme et al, 1997; van Damme et al., 1994) and soft tissue expansion was independent of the presence of scarred tissue. On the other hand, skin expansion decreased the limiting effect of scar tissues on restriction of mid-facial growth (Edington, Mooney, Losken, Hurwitz, & Siegel, 1998), implying the option of expanding compromised soft tissues. On the other hand, despite the presence of scars might not be an

absolute contraindication for expansion, but it must be taken as part of consideration that it may compromised soft tissues with reduced expanding capacity to normal tissue (Fang, Zhou, & Yang, 2013).

Based from the previous reported case series (Kaner & Friedmann, 2011), retreatment with soft tissue expanders was not carried out. All other patients experienced normal healing without any complications. Final expansion of the vestibular mucosa was achieved, and all the expanders reached their final size, limiting the amount of gain keratinized mucosa. The quality of the expanded tissues was lining and masticatory mucosa. The authors explained that the possibility of associated with applied expansion technique, as all the expanders were placed in the vestibule and thus were only surrounded by alveolar mucosa. Following expanders removal, two recipient sites showed sign of underlying bone resorption. This observation did not associated with adverse complications, and the corresponding areas have been successfully augmented.

Vertical and horizontal bone augmentation were performed with either autogenous or synthetic block grafts. No periosteal releasing incisions were needed to achieve primary soft tissue closure over the bone graft. Except in the two patients who experienced soft tissue perforation and pre-mature removal of the expanders. Post-operative healing of the grafts was uneventful. In the meanwhile of implant placement, all bone grafts were successfully healed and soft tissue quantity was sufficient to passively close the mucosal flaps. Thorough evaluation of the amount of keratinized and non-keratinised soft tissue is important to plan accordingly the soft tissue expansion prior for bone or implant surgery (Park et al., 2013). Final clinical follow-up, no

complications were reported and all implants were intact. Overall, it can be summarize that soft tissue expansion prior to bone augmentation may reduce the risk of mucosal dehiscence with subsequent bone graft exposure. Additional randomized controlled clinical trials, with adequate sample size and long-term follow-up are needed to confirm these findings.

In a published case report by Park et al. (2013), sub-periosteal hydrogel osmotic expanders were used prior to vertical bone augmentation in severely resorbed mandibular ridges. They were left in-situ for 3 or 6 weeks. At the time of bone grafting, tension-free and complete closure of the augmented bone with overlying soft tissues was achieved. After healing, the grafted bone was hard and intact clinically, and implants could be placed without any complications. Similar positive outcomes were documented with pre-augmentation of soft tissue expansion, in a patient with significant bone resorption in the posterior area of the mandible (von See et al., 2010).

Another application of soft tissue expander is the repair of lip and palate clefts. *In vivo* studies show variable outcomes using the soft tissue expanders. In a rabbit cleft lip model, an extensive soft tissue was generated by expansion of the labial surface area, resulting in reduced post-operative lip pressure and improved mid-facial growth (Edington et al., 1998). Meanwhile, in a cleft lip and palate model in cats (van Damme et al., 1997) even though soft tissue expansion of the palatal mucosa were available, retardation of transverse growth was reported as an iatrogenic side effect from active expansion. In addition, previous study reported (van Damme et al., 1994), active expansion resulted in palatal bone resorption.

Kobus (2007) conducted a clinical study using hydrogel soft tissue expanders as an adjunctive in two-stage repair of cleft palate in children over 15 months. The clinician intended to limit their palatal scarring and therefore preserve maxillary growth. Out of nineteen children enrolled in the study, seven resulted with fistulae despite the adjunctive application of soft tissue expanders. The high rate of fistulae was explained by the lack of silicon coating around the surface of osmotic expanders which resulted in super-quick expansion with concomitant wound dehiscence. A study by Swan, Goodacre, Czernuszka, and Bucknall (2008) indicated the quick expansion technique in children may cause complications as well as soft tissue expanders tend to expand equally in all directions and may not be feasible in a confined area like the palate. Swan et al. (2008) also recommended that directionally dependent expansion must be developed to allow spontaneous swelling in transverse direction only.

A novel anisotropic self-inflating hydrogel tissue expander was suggested in improving future clinical applications of soft tissue expansion in cleft palate defects, eyelid and nasal tip reconstruction. It is a novel expander, based on methyl methacrylate and vinyl pyrrolidone designed to display anisotropy, and exhibit a capacity for considerable expansion together with a

controlled modifiable expansion rate (Swan et al., 2011). Anisotrophy was induced through compression of hydrogel copolymer at elevated temperatures, and expansion rate was controlled by incorporation of a semi-permeable silicone membrane, *in vitro.*

The efficacy of this novel expander was tested *in vivo*, by sub-periosteal implantation in hard palates of pigs (Swan et al., 2012). Uncoated and silicone-coated expanders were compared 6 weeks post-expansion. In comparable to all published findings in the literature, uncoated devices resulted in rapid expansion causing muco-periosteal ulceration, while coated expanders outplayed a more controlled expansion. Coated expanders revealed a significant increase in soft tissue volume without any evidence of acute inflammation. Formation of soft tissue capsule was present around the expanders, and expander-mediated erosion of palatal bone could be observed. Despite the promising results, clinical research is needed to investigate the outcomes of anisotropic expansion in specific intra-oral applications.

2.3 Anatomy and Histology of skin

2.3.1 The epidermis

Light and electron microscope studies involving expanded tissue from both animal and humans have been extensively reported (Argenta, Marks, & Pasyk, 1985; Austad, Pasyk, Mcclatchey, & Cherry, 1982; Pasyk et al., 1988; Pasyk, Austad, & McClatchey, 1982; Shan & Baker, 1991). In general human and animal soft tissue respond in a similar manner during conventional controlled tissue expansion (Johnson et al., 1993). The skin is the largest organ of the body, with 15% of the total adult body weight. It performs many vital functions, including protection against external physical, chemical and biologic assailants, including prevention of excess water loss from the body and a role in thermoregulation. The skin is continuous, with the mucous membrane lining the body's surface (Kanitakis, 2002).

The integumentary system is formed by the skin and its derivative structures. The skin consist of three layers: the epidermis, the dermis, and subcutaneous tissue. The outermost area, the epidermis, consists of a specific constellation of cells known as *keratinocytes*, play roles in synthesize keratin, a long, threadlike protein with a protective role. The middle layer, dermis, mainly made up of the fibrillar structural protein identified as collagen. The dermis lies on the subcutaneous tissue, or a *panniculus*, which contains small lobes of fat cells known as lipocytes. The thickness of these layers varies, depending on the geographic location on the anatomy of the body. The eyelid presented with the thinnest layer of the epidermis, less than 0.1 mm, while palms and soles of the feet have the thickest epidermal layer (1.5 mm). The dermis is thickest on the back, 30-40 times as thick as the overlying epidermis (James, Berger, & Elston, 2006)

The epidermis (Figure 2.7) is stratified, squamous epithelium layer that is composed primarily of two types of cells: keratinocytes and dendritic cells. The keratinocytes differ from the "clear" dendritic cells by possessing intracellular bridges and ample amounts of stainable

cytoplasm (Murphy, 1997). The epidermis harbors a number of other cell populations, including melanocytes, Langerhans cells, and Merkel cells, but the keratinocyte cell comprises the majority of the cells. The epidermis is divided into four layers according to keratinocyte morphology and position as they differentiate into horny cells, including the basal cell layer (stratum germinativum), the squamous cell layer (stratum spinosum), the granular cell layer (stratum granulosum), and the cornified or horny cell layer (stratum corneum) (Murphy, 1997). The lower three layers that constitute the living, nucleated cells of the epidermis are sometimes referred to as the stratum malpighii and rete malphigii (Chu, 2008).

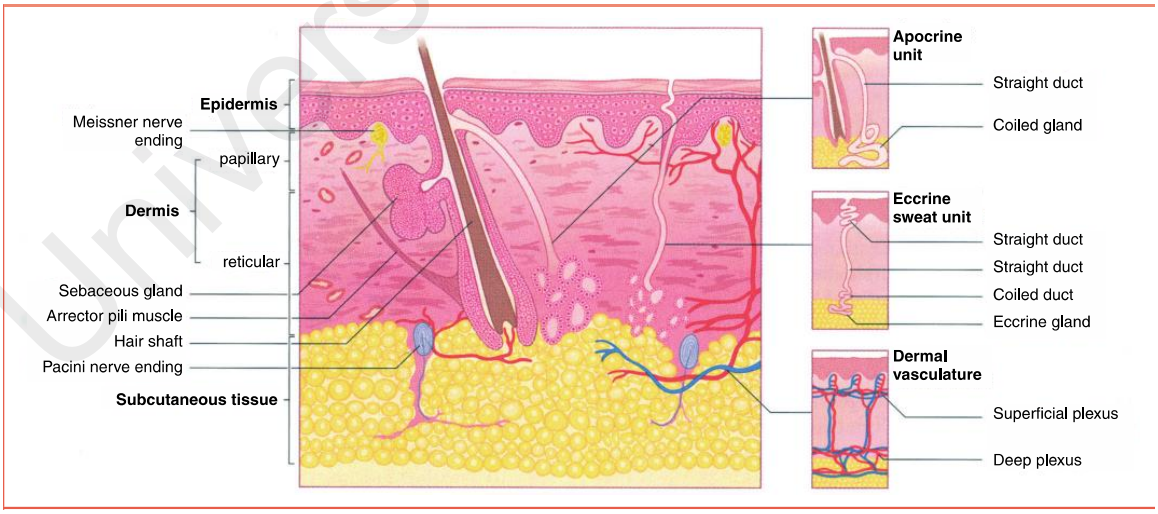

Figure 2.7: Cross-section of Skin and Panniculus.(Goodwin, 2011).

The epidermis is continually renewing layer and give rise to derivative structures including pilosebaceous apparatus, nails and sweat glands. The basal cells of the epidermis undergo proliferation cycles that provide renewal of the outer layer. The epidermis is a dynamic tissue in which cells are constantly in disorganized manner, as differing individual cell populations pass not only one another but also melanocytes and Langerhans cells as they migrate towards the skin surface (Chu, 2008).

At least 80% of cells in the epidermis are the ectodermal derived keratinocytes. The differentiation process that occurs as the cells migrate from the basal layer to the surface of the skin results in keratinization (keratinocyte passes through a synthetic phase and then a

degradative phase) (Chu, 2008). In the synthetic phase, the cell builds up a cytoplasmic supply of keratin, a fibrous intermediate filament arranged in an alpha-helical coil pattern that serves as part of the cell's cytoskeleton. Bundles of these keratin filaments converge on and terminate at the plasma membrane forming the intercellular attachment, known as desmosomes. During the degradative phase of keratinization, cellular organelles are lost, the contents of the cell are consolidated forming mixture of filaments and amorphous cell envelopes, and the cell finally become horny cell or corneocyte. The process of maturation resulting in cell death (terminal differentiation) (James et al., 2006).

The basal layer, also known as the stratum germinativum, contains column-shaped keratinocytes that attach to the basement membrane with their long axis perpendicular to the dermis. These basal cells form a single layer and adhere to one another as well as to more superficial squamous cells through desmosomal junctions (Murphy, 1997). The prominent features of the basal cells are their dark-staining oval or elongated nuclei and the presence of melanin pigment transferred from adjoining melanocytes. The basal layer is the primary location of mitotically active cells in the epidermis that give rise to cells of the outer epidermal layers. On the other hand, not all basal cells have the potential to divide (Jones, 1996). Epidermal stem cells in the basal layer are clonogenic cells with long lifespan that undergo slow and progressive cell cycle in a normal conditions. In condition like wounds, it can increase the number of cycling cells in the epidermis by stimulating division of stem cells. DNA damage by carcinogenic agents may mutate cell proliferation and affect the rate of cell division. Migration of a basal cell from the basal layer to the cornified layer in humans take at least 14 days, and the transit through the cornified layer to the outermost epidermis requires 14 days (Chu, 2008).

Overlying the basal cell layer is a layer of the epidermis with 5-10 cell thickness, the squamous cell layer or stratum spinosum (Murphy, 1997). The squamous layer is composed of a variety of cells with different shape, structure, and subcellular properties. Suprabasal spinous cells, are polyhedral in shape and have a rounded nucleus, while cells on the upper spinous layers are larger in size, become flatter as they are pushed toward the skin surface and contain lamellar granules (Chu, 2008). Lamellar granules are membrane-bound organelles containing glycoproteins, glycolipids, phospholipids, free sterols and a number of acid hydrolases (e.g. lipase, proteases). The presence of hydrolytic enzymes indicates that the lamellar granules are lysosome type. Lamellar granules are active in cells at the interface between the granular and cornified layers, by deliver the precursors of stratum corneum lipids into the intercellular space. Intercellular space between spinous cells are bridged by abundant desmosomes that promote mechanical coupling between cells of the epidermis and provide resistance to physical stresses. Organized concentrically around the nucleus, keratin filaments in the cytoplasm are bound to desmosomal plaques at one end and remain free at the end of the nucleus (Murphy, 1997).

The most superficial layer of the epidermis containing living cells, the granular layer of stratum granulosum, composed of flattened cells holding abundant keratohyline granules in their cytoplasm. These cells are responsible for further synthesis and modification of proteins involved in keratinization (Chu, 2008). The granular layer varies in thickness in proportion to that of the overlying horny cell layer. A thin granular layer can lead to extensive parakeratosis, the nuclei of keratinocytes persist as the cells move into the stratum corneum resulting in psoriasis (Murphy, 1997).

Horny cells of the cornified layer provide mechanical protection to the underlying epidermis and a barrier to prevent water loss and invasion by foreign substances (Jackson, Williams, Feingold, & Elias, 1993). The corneocytes are rich in protein and low in lipid content and surrounded by a continuous extracellular lipid matrix (Chu, 2008). The large, flat, polyhedral-shaped horny cells have lost their nuclei during terminal differentiation and considered lifeless. (Murphy, 1997). The physical and biochemical properties of cells in the cornified layer vary in accordance with position in order to promote desquamation moving outward. Cells in the middle layer have higher capacity for water-binding compared to deeper layers because of high concentration of free amino acids found in the cytoplasm. The deep cells are more compact and exhibit greater array of intercellular attachments. Desmosomes undergo proteolytic degradation as the cells progress outward, contributing to the shedding of corneocytes during desquamation (Haake & Hoolbrook, 1999).

The epidermis is a regenerative tissue that maintaining its constant number of cell as well as regulate the interactions and junctions between epidermal cells. Epidermal morphogenesis and differentiation is regulated by the underlying dermis, in maintaining postnatal structure and function. The epidermal-dermal interface also play role in the development of epidermal appendages. The maintenance of a constant epidermal thickness depends on intrinsic properties of epidermal cells including the ability to undergo apoptosis. Apoptosis follows an orderly pattern of morphologic and biochemical changes resulting in cell death without injury to neighboring cells. The homeostatic mechanism is regulated by a number of cellular signaling molecules including hormones, growth factors, and cytokines. In the skin, apoptosis is important in developmental remodeling, regulations of cell numbers, and defense. Terminal differentiation

is type of apoptosis in order to convert keratinocyte into protective corneocyte (Haake & Hoolbrook, 1999). Disruption in dynamic equilibrium maintaining constant epidermal thickness resulted in condition like psoriasis, dysregulation of apoptosis often seen in tumors of the skin.

The melanocytes is a dendtritic, pigment-synthesizing cell derived from neural crest and confined in the skin mainly at the basal layer (Chu, 2008). Melanocytes are responsible for the production of the pigment melanin and its transfer to keratinocytes. Melanin is round in shape, membrane-bound organelle known as melanosome. Melanosomes moved to the end of the melanocyte processes that lie close to the skin surface and transferred to keratinocytes. Increased in ultraviolet light exposure stimulates an increase in melanogenesis and corresponding increase in melanosome transfer to keratinocytes as the melanosomes aggregate toward the superficial side of the nucleus. This results in tanning of the skin, increase the cell's ability to absorb light and thus protect genetic information in the nucleus from damaging radiation (Haake & Hoolbrook, 1999).

Merkel cells are oval-shaped, slow-adapting, type I mechanoreceptors located in sites of high tactile sensitivity that attached to basal keratinocytes by desmosomal junctions. Merkel cells are found in the digits, lips, regions of the oral cavity, and outer root sheath of the hair follicle and sometimes assembled into specialized structures, tactile discs or touch domes. Small deformations of adjoining keratinocytes are stimulus to cause Merkel cells to secrete a chemical signal in generating action potential in the adjoining afferent neuron, and relays signal to the brain (Haake & Hoolbrook, 1999).

The interface between the epidermis and dermis is formed by a porous basement membrane zone (James et al., 2006). Basal keratinocytes are the most important components of structures of the dermal-epidermal junction. The basal lamina is a layer synthesized by basal cells of the epidermis consist of type IV Collagen. The plasma membranes of basal cells area attached to the basal lamina by rivet-like hemidesmosomes that distribute tensile or shearing forces through the epithelium. This junction act as support for the epidermis, establishes cell polarity and direction of growth, directs the organization of the cytoskeleton in basal cells, provide developmental signals, and functions as semipermeable barrier between layers (Murphy, 1997).

The skin adnexa are a group of ectodermal derived appendages, including eccrine and appocrine glands, ducts and pilosebaceous units that originate as down growths from the epidermis during development. All adnexal structures are capable of reepithelization and it occurs more rapidly after injury (James et al., 2006). Eccrine sweat glands involved in the regulation of heat and are most abundant on the soles of the feet (Murphy, 1997). The sweat glands originate as a band of epithelial cells growing downward from the epidermal ridge and modified during development to generate three composite parts of the eccrine sweat unit, which are the intraepidermal spiral duct, the straight dermal portion, and the coiled secretory duct (James et al., 2006). The secretory coil of the eccrine unit lies deep in the dermis or within the superficial panniculus and composed of glycogen-rich clear secretory cells, dark mucoidal cells, and myoepithelial cells specialized in contractile properties (James et al., 2006).

Eccrine glands are primarily involved in thermal regulation and involved in scent release (Murphy, 1997). The apocrine sweat glands in human are confined mainly to the regions of the axillae and perineum, and unlike eccrine and apoeccrine glands, they do not open directly into the cells from which the dermal papilla is formed. Differentiation occurs at the lower portion of the hair follicle forming the hair, the cuticle, and the two inner root sheaths. Differentiation in the upper segments of the follicle producing the hair canal in the upper dermis, through the epidermis, and opening to the surface prior to the time that the growing hair cone reaches the upper follicle.

The sebaceous gland forms from a bud in the fetal hair follicle. Along the same side of the follicle but below the sebaceous gland, another bud develops into an attachment for the arrector pili muscle. The *arrector pili* (AP) are a smooth muscle bundle that attaches to the external root sheath of the follicle. On the opposite side of the follicle (Figure 2.8), a third bud forms above the plane of the sebaceous gland and develops into the apocrine gland. Proliferating cells in the hair bulb, called *matrix cells*, are responsible for the production of the hair shaft as well as the inner and outer root sheaths (James et al., 2006). Hair growth occurs in a cyclical manner, but each follicle functions as an independent unit.

The hair growth cell cycle is composed of three stages (Figure 2.9): anagen, catagen, and telogen. *Anagen*, the active growth stage, typically lasts approximately three to five years on the scalp, during which hairs grow at a rate of about 0.33 mm per day. The length of the anagen phase decreases with age and decreases dramatically in individuals with alopecia. *Catagen* usually lasts about two weeks and is a period of involution resulting in club hair formation after

many cells in the outer root sheath undergo apoptosis. The resting phase, *telogen*, lasts about three to five months on the scalp, and hairs in this stage are eventually pushed out by the growing anagen hair shaft. Two secreted molecules that may have important roles in hair follicle development and cycling are the insulin-like growth factor 1 and fibroblast growth factor 7. In mice, both are produced by the dermal papilla and have receptors predominantly in overlying matrix cells. Hormonal factors controlling hair growth include estrogens, thyroid hormones, glucocorticoids, retinoids, prolactin, and growth hormone. The hormones with the most impressive effect are the androgens: testosterone and its active metabolite, dihydrotestosterone, which act through androgen receptors in the dermal papilla.

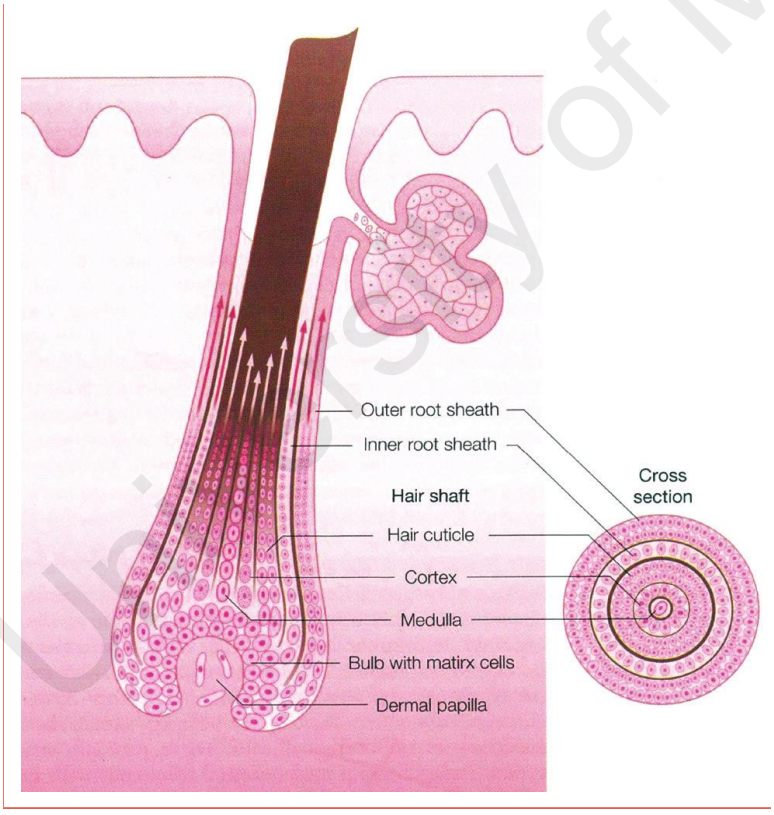

Figure 2.8: Hair Follicle structure (Goodwin, 2011).

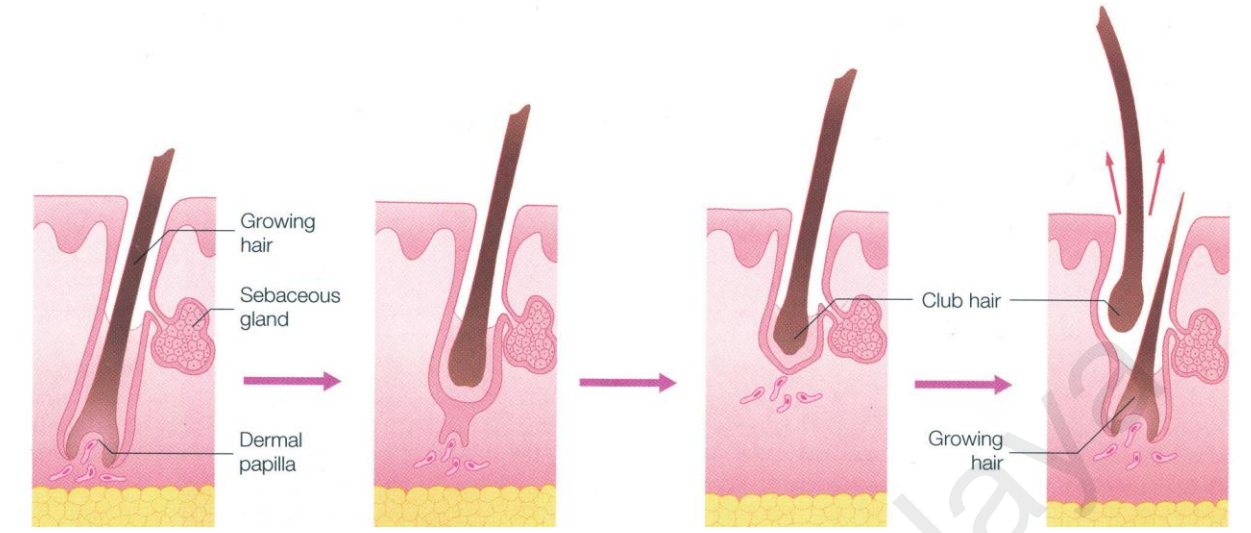

Figure 2.9: Phases of hair growth (Goodwin, 2011).

Sebaceous glands are found in greatest number on the face and scalp but are present on nearly all other locations of the body (James et al., 2006). Cells of the sebaceous glands contain abundant lipid droplets known as *sebum* in their cytoplasm and are arranged into lobules off the upper segment of the hair follicle. Basaloid germinative cells surrounding the lobule give rise to the lipid-filled cells, which are then expelled into the infundibular segment of the hair follicle via the sebaceous duct.

2.3.2 The dermis

The *dermis* is an integrated system of fibrous, filamentous, and amorphous connective tissue that accommodates stimulus-induced entry by nerve and vascular networks, epidermally derived appendages, fibroblasts, macrophages, and mast cells. Other blood-borne cells, including lymphocytes, plasma cells, and other leukocytes, enter the dermis in response to various stimuli

as well. The dermis comprises the bulk of the skin and provides its pliability, elasticity, and tensile strength. It protects the body from mechanical injury, binds water, aids in thermal regulation, and includes receptors of sensory stimuli. The dermis interacts with the epidermis in maintaining the properties of both tissues. The dermis does not undergo an obvious sequence of differentiation that parallels epidermal differentiation, but the structure and organization of the connective tissue components are predictable in a depth-dependent manner. The matrix components, including collagen and elastic connective tissue, also vary in a depth-dependent manner and undergo turnover and remodeling in normal skin, in pathologic processes, and in response to external stimuli (Chu, 2008).

The constituents of the dermis are mesodermal in origin except for nerves (melanocytes, derive from the neural crest). Until the sixth week of fetal life, the dermis is merely a pool of dendritic-shaped cells full of acid-mucopolysaccharides, which are the precursors of fibroblasts. By the 12th week, fibroblasts are actively synthesizing reticulum fibers, elastic fibers, and collagen. A vascular network develops and fat cells have appeared beneath the dermis by the 24th week. Infant dermis is composed of small collagen bundles, whereas the adult dermis contains thicker bundles of collagen. Many fibroblasts are present in the infant dermis, but few persist in adulthood (James, Berger, & Elston, 2006). The principal component of the dermis is *collagen*, a fibrous family of proteins with at least 15 genetically distinct types in human skin. A major structural protein, collagen is found in tendons, ligaments, the lining of bones, and the dermis. Collagen is a major stress-resistant material of the skin. Elastic fibers, on the other hand, play a role in maintaining elasticity but do very little to resist deformation and tearing of the skin. Collagen fibers exist in a constant state of flux, being degraded by proteolytic enzymes called *spare collagenases* and replaced by new fibers. Fibroblasts integrate the procollagen molecule, a

specific helical polypeptide chain. Then, the cell secretes the fibroblasts, and they assemble into collagen fibrils. The amino acids glysine, hydroxyproline, and hydroxy-lysine highly enrich collagen. The fibrillar collagens found in the skin comprise the major group and are the most abundant proteins in the body (Goodwin, 2011).

The major constituent of the dermis is type I collagen, loosely positioned fibers are found in the papillary and adventitial dermis, whereas hefty collagen bundles are noted in the reticular dermis. Type IV collagen is found in the basement membrane zone, and the major structural component of anchoring fibrils is collagen type VII, which is produced primarily by keratinocytes The elastic fiber differs both structurally and chemically from collagen and consists of two components: protein filaments and *elastin,* an amorphous protein. The fibroblast fuses elastic fiber to the extracellular matrix of the dermis, which is composed of glycosaminoglycans. The fibers are fine in the papillary dermis and coarse in the reticular dermis. Hyaluronic acid is a minor component of the normal dermis but is the major mucopolysaccharide that accumulates in pathologic states (James et al., 2006).

The dermal vasculature is made up of two intercommunicating *plexuses*: the *subpapillary* or *superficial plexus* composed of postcapillary venules found at the junction of the papillary and

reticular dermis and the *lower plexus* at the dermal-subcutaneous interface. The dermal papillae are supplied by capillaries, end arterioles, and venules of the superficial plexus. The deeper plexus is supplied by larger blood vessels and is more complex surrounding adnexal structures. Blood flow in human skin fluctuates significantly in response to thermal stress because of the regulation of the preoptic-anterior hypothalamus (Goodwin, 2011).

Nerve bundles, together with arterioles and venules, are found in great quantity in neurovascular bundles of the dermis (James et al., 2006). Meissner corpuscles, found in the dermal papillae, help to mediate touch and are found predominantly on the ventral sides of the hands and feet. Meissner corpuscles occur in greater abundance on the hands, with greatest concentration in the fingertips. Vater-Pacini corpuscles are large nerve-end organs that generate a sense of pressure and are located in the deeper portion of the dermis of weight-bearing surfaces and genitalia. They also are found commonly in the nipple and anogenital region. Pain, temperature, and itch sensation are transmitted by unmyelinated nerve fibers that end around hair follicles and the papillary dermis. Vasoconstriction is regulated by the postganglionic adrenergic fibers of the autonomic nervous system. This system regulates the apocrine gland secretions and the contraction of AP muscles of hair follicles. Eccrine sweat secretions, on the other hand, are mediated by cholinergic fibers (Goodwin, 2011).

Embryologically, toward the end of the fifth month fat cells begin to develop in the subcutaneous tissue. These lobules of fat cells or lipocytes are separated by fibrous septa made up of large blood vessels and collagen. The panniculus varies in thickness depending on the skin site. Considered an endocrine organ, the subcutaneous tissue provides the body with buoyancy and functions as a storehouse of energy. Hormone conversion takes place in the panniculus, converting androstenedione into estrone by aromatase. Lipocytes produce leptin, a hormone that regulates body weight by way of the hypothalamus (James et al., 2006).

2.4. Protein Markers.

2.4.1. Nestin

Many cell reactions directly involve the cytoskeleton with intermediate filaments (IF) along with actin-containing microtubules make up the three major classes of the cytoskeleton and further classified according to various criteria (Fuchs & Weber, 1994) . There are over 70 members in the IF protein superfamily , and divided into six or seven classes based on gene structure and sequence comparisons throughout rod domains, and terminal head and tail domains. The rod domain promotes coiled-coil interactions between two individual IF proteins to initiate formation of 10-nm diameter filaments. Flanking this rod domain are the N- terminal head and C- terminal tail domains vary in size sequence of IF classes (Hermann et al., 1996).

Intermediate filaments (IF) proteins can self-assemble into cytoskeleton filaments, with diameter of 10-20 nm on visualization by negative staining and electron microscopy (Hermann et al., 2007). All major types of IF polymers identified as mechanical scaffolds in differentiated cell types in situ (Coulombe, Ma, Yamada, & Wawersik, 2001). Defects in the polymer structure results in fragility states that cause loss of cellular integrity following exposure to shearing forces.

Nestin, an acronym for "neuroectodermal stem cell protein", is a class 6 intermediate filament protein (classes 1 and 2 are acidic and basic keratins, class 3 includes vimentin, desmin, glial fibrillary acidic protein, and peripherin; class 4 includes neurofilaments and internexins; class 5 includes nuclear laminins) (Lendahl, Zimmerman, & Mckay, 1990b). Nestin was discovered in 1985 as an antigen recognized by monoclonal antibody Rat-401 (Hockfield & McKay, 1985). This antibody was obtained by immunization of mice with proteins extracted from the spinal cord of 15-old-day rat embryos. The nestin gene was characterized in 1990 (Lendahl et al., 1990b). The positions and lengths of its introns are highly conserved: the exon-intron structures of nestin genes are identical in mice, rats and humans (Michalczyk & Ziman, 2005). Two introns are homologous to their counterparts in neurofilament genes and located in same position including similar sequence. This suggest that nestin gene and three neurofilament genes arose from a common precursor by gene duplications.

The nestin protein consist of more than 1600 amino acids with molecular weight 198kD and it is analogous to proteins of other intermediate filaments that expressed in dividing cells during early stages of development in the CNS and PNS, and in myogenic and other tissues (Bernal & Arranz, 2018). After differentiation, nestin is downregulated and replaced by tissue-specific IF proteins. However, nestin expression is reinduced in adults during pathological situations, such as during formation of a glial scar after CNS injury or regeneration of injured muscle tissue. The distribution and expression of nestin is mitotically active cells suggest it plays a role in regulation of the assembly and disassembly of Ifs (Bernal & Arranz, 2018).

In view of its short N- terminal, nestin can only form heterodimers and heterotetramers by binding with other intermediate filament proteins. The processes of assembly and disassembly are regulated by phosphorylation of a threonine residue at position 316 in the amino acid sequence (Sahlgren et al., 2001). A low level of phosphorylation is associated with the process of filament assembly, meanwhile, three-fold increase in the level of nestin phosphorylation during mitosis leads to dissociation of intermediate filaments. The key role in nestin phosphorylation is placed by cdc2 kinase together with cyclin B, key in regulating transfer from the G_2 to the M phase of the cell cycle. The long C terminal involve in the interaction of intermediate filaments with microfilaments and microtubules. In addition, the C terminal contains a large quantity of serine, for additional phosphorylation site. Phosphorylation of serine may alter the configuration of side branches and affect the binding of nestin with other components of cytoskeleton. Thus,

nestin has been suggested to take part in stabilizing cell structure and coordinating changes in intracellular dynamics including cell division and migrations (Michalczyk & Ziman, 2005).

The nervous system is divided into two specific compartments, the central nervous system (CNS) and peripheral nervous system (PNS). It is characterized by a very poor regenerative capacity, which is limited to the PNS and, more specifically structure that forms a highly complex network throughout the body to provide motor and/or sensory innervations to target organs (Fornaro et al., 2008). Histologically, two main elements characterize the PNS: the functional unit, or parenchyma; and the stroma. The parenchyma consists of nerve fibers formed by axons from motor and sensory neurons, and the surrounding Schawnn calls. In addition, the dorsal root ganglia (DRG), which contain sensory neurons, also part of the PNS; these sensory cell bodies have axons that extend to dendrites in both the PNS and spinal cord (Barnat et al., 2010). Injury to mammalian peripheral axons induces a variety of metabolic changes in the neuronal cell body that support the process of axonal regeneration. Nerve regeneration results from a balanced interaction of Schawnn cells, the environment, the extracellular matrix and the regenerating axons (Liem & Messing, 2009).

According to the literature, nestin is regard as a marker for "neural stem/ progenitor cells" (NSPC) (Fukuda, Kato, Tozuka, & Yamaguchi, 2003; Hendrickson, Rao, Demerdash, & Kalil, 2011; Lariviere & Julien, 2003; Michalczyk & Ziman, 2005; Podgornyi, 2006; Savchenko, Andreeva, & Dmitrieva, 2005). First , Nestin is observed in cells in nervous tissue forming during embryonic period ontogenesis (Dahlstrand, Lardelli, & Lendahl, 1995; Hockfield &

McKay, 1985). The number of dividing cells in the mammalian brain decreases significantly immediate after birth. Proliferation continues in two area, where mitotic activity noted in individuals (Altman & Das, 1965; Cameron & McKay, 2001; Fukuda et al., 2003; Gage, 2002). Second, Nestin is expressed in both neuronal (Fukuda et al., 2003; Parlakian, Paulin, Izmiryan, Xue, & Li. Z., 2016) and glial cells (Hendrickson et al., 2011; Nakamura, Xi, & Hua, 2003; Savchenko et al., 2005), as well as in their common precursors (Andressen, Stocker, & Klinz, 2001; Cattaneo & McKay, 1990; Hockfield & McKay, 1985). Third, various forms of damage to the brain and spinal cord are followed by the appearance of nestin-immunopositive cells in these areas (Holmin, von Gertten, & Sandberg-Nordqvist, 2001; Kaya, Mahmood, & Li, 1999; Nakagawa, Miyamoto, & Janjua, 2004). It was suggested that it involves replacement of dead cellular elements by new cells containing nestin. Fourth, stem cells (embryonic stem cells for definitive tissues) transplanted into the nervous system start to express nestin (Li & Chopp, 1999). Fifth, cells of various nervous system tumors (neurocytomas (You, Kim, & Im, 2005), neuroblastomas (Biagiotti, D'Amico, & Marzi, 2006), gliomas (Kambara, Okano, Chiocca, & Saeki, 2005), including glioblastomas, ependymomas (Almqvist, Mah, & LendHL, 2002) and Schwannomas (Parfitt, McLean, & Joseph, 2006)) also express nestin. It also suggest that tumors show same gene expression as undifferentiated cells (Dahlstrand, Collins, & Lendahl, 1992).

On the other hand, few authors suggested that not all nestin-immunopositive cells are members of the NSPC population, some being differentiated cells (astrocytes), which is without a specific reasons start to synthesize embryonic protein (Clarke, Shetty, Bradley, & Turner, 1994; Holmin et al., 2001; Khorzhevskii, Otellin, & Groigorev, 2004). In addition, nestin is also expressed in other organs and tissues (the retina (Kohno, Sakai, & Kitahara, 2006; Wislet-

Gendebien, Hans, & Leprince, 2005), striated muscle (Lendahl, Zimmerman, & McKay, 1990), cardiac muscle, skin (Medina, Kataoka, & Takaishi, 2006) and its derivatives (Hoffman, 2006), teeth (About, Laurent-Maquin, Lendahl, & Mitsiadis; Graham, Cooper, & Cassidy, 2006), liver (Forte, Minieri, & Cossa, 2006; Li & Chopp, 1999), the pancreas (Ueno, Yamada, & Watanabe, 2005), the kidney (Zou, Yaoita, & Watanabe, 2006), the testicles (Frodjman & Pelliniemi, 1997), the adrenals (Toti, Regoli, & Nesi, 2005), and others). Based on the evidence presenting nestin-containing cells are pluripotent and not exclusively origin of neuroepithelial, and as conclusion nestin cannot be unambiguously interpreted as a marker of NSPC.

Nestin expression in mice is observed during early stages of development, starting from day 7 of intrauterine life, and occurs in several proliferative zones of the central nervous system: in embryos during 10.5- day stage, nestin is expressed in cells in both the rostral and caudal parts of the neural tube (Dahlstrand et al., 1995); Nestin appears in the rat spinal cord in 11-day embryos and continues to be detectable in postnatal day 6 (Hockfield & McKay, 1985); nestin-immunopositive cells is detected in the brainstem of rat embryos at the 15-day stage (Zhou, Sari, & Powrozek, 2003); nestin is present in the cerebellum of animals (mice, rats) from 15.5 days of intrauterine life to 21 days postnatal day (Dahlstrand et al., 1995), and in the endbrain from day 10 of Intrauterine life (IUL) (mice).

Nestin can be seen in several cell types at the early stages of embryonic development: cells of the radial glial (Hockfield & McKay, 1985), cells of the ventricular zone, common (neuronal and gliocytic precursors), and neuroepithelial cells (Taylor, Sater, & French, 2001).

The transition from proliferation to the post-mitotic state is accompanied by a rapid decrease in the quantity of nestin mRNA (Dahlstrand et al., 1995). As differentiation of nervous tissue proceeds, nestin synthesis decreases (Lothian & Lendahl, 1997), and fibrillary glial acidic protein (FGAP) and neurofilament proteins starts to be expressed in differentiating astrocytes and neurons respectively. There is a progressive decrease in nestin expression during the postnatal period. Normally nestin is not detected in the brains of adult animals, although nestin-immunopositive cells have been described in the following areas: the subventricular zone (SVZ) of the lateral ventricles (Ernst & Christie, 2005) and the dentate gyrus (DG) of the hippocampus (Cameron & McKay, 2001; Fukuda et al., 2003). These area were identified as germinatives zones of the brain, as neurogenesis is believed to continue in these areas after birth.

Nestin-immunopositive cells are seen in the lateral wall of the lateral ventricles: ependymocytes with long processes, morphologically similar to radial gliocytes, and round cells in the subependymal zone. The number of nestin-expressing cells in the SVZ of the lateral ventricles is significantly smaller than in the SVZ of the third ventricle of the brain (Ernst & Christie, 2005). Thus nestin expression does not correlate with the mitotic activity of cells in the SVZ of the third ventricle. It has been suggested that the SVZ contains a temporary population of proliferating cells, which give rise to nestin-expressing cells, which later differentiate into neurons and gliocytes (Alvarez-Buylla, Seri, & Doetsch, 2002).

Various types of CNS damage are followed by reactive neurogenesis (the formation of new neurons) (Parent, 2003) and glial elements (Horner & Gage, 2000) to replace those lost in

response to the damaging factor. Increased nestin expression is seen in a number of pathological states, including ischemia (Duggal, Schmidt-Kastner, & Hakim, 1997), traumatic brain damage (Itoh, Satou, Hashimoto, & Ito, 2005), inflammation (Cameron & McKay, 2001), epilepsy (Ying, Gonzalez-Martinez, Tilelli, Bingaman, & Najm, 2005), experimental depolarization (Holmin et al., 2001).

The neuronal cytoskeleton plays an essential role throughout regeneration by providing the basis of motility, structure and stabilization in the newly formed axons (Willis et al., 2005). The fact that IFs are involved in the process of nerve regeneration has been widely acknowledged, but few have focused on neuronal cell bodies, while the role of IFs is far from being completely understood. Data concerning the involvement of IFs in axonal regeneration have mainly been obtained from mouse knockout models or mice overexpressing mutant forms of IFs. Indeed, overexpression of a dominant negative mutant form of peripherin led to the disruption of the IF network in sensory neurons in vivo and showed that peripherin IFs are required for collateral sprouting (Belecky-Adams et al., 2003).

2.4.2 TrKβ

The cutaneous endocrinology is a rapidly expanding area spiced with exciting and high quality basic and clinical research. Attention focused on the classical endocrine hormones and signaling pathways such as the equivalent of the hypothalamus-pituitary-adrenocortical axis (O'Kane, Murphy, & Kirby, 2006). Its central protagonist's corticotropin releasing hormone and adrenocorticotropic hormone for example are produced in the skin, and most recent discoveries demonstrate their full capacity to induce cortisol-production by skin cells (Ito, Ito, & Paus, 2005). However recently growth factor families, which specialized in non-skin related contexts are rediscovered as endocrine hormones regulating neuroendocrine functions in skin. The neurotrophin (NT) family has acquired a central position. Its cardinal protagonist nerve growth factor (NGF) has long been acknowledged as an auto- and paracrine growth factor for virtually

all skin cells (Botchkarev, Yaar, Peters, et al., 2006). Its additional functions including immunmodulator, a pain sensitizer and a stress-mediator in skin and contribute to the rise of its family members in scientific appreciation (Anand, 2004).

Neurotrophins consist of a family of four related polypeptide growth factors: Nerve growth factor (NGF) (Levi-Montalcini & Cohen, 1960), brain-derived neurotrophic factor (BDNF) (Barde, Edgar, & Thoenen, 1982), neurotrophin-3 (NT-3) (Hohn, Leibrock, Bailey, & Barde, 1990) and neurotrophin -4/5 (NT-4/5) (Berkemeier, 1991). The NTs are synthesized as precursors known as proneurotrophins and cleaved to produce mature proteins. They transmit most of their known actions via two classes of transmembrane receptors that may interact with a low-affinity pan-NT receptor $p75^{NTR}$ and high-affinity NT receptors of the tyrosinekinase (Trk) family TrkA-C (Botchkarev, Botchkareva, Peters, & Paus, 2004). Interestingly, proneurotrophins bind with high- affinity to $_{75}NTR$ (Lu, Pang, & Woo, 2005): mature NTs bind preferentially to Trk receptors, indicating that pro and mature NTs have their own preferred cognate receptors.

The $p75^{NTR}$ belongs to the tumor necrosis factor (TNF)-superfamily, such as TNF receptor and Fas, proteins known to share a conserved sequence in their cytoplasmic domain, which can signal cell death upon activation (Locksley, Killeen, & Lenardo, 2001). The $p75^{NTR}$ was the first NT receptor to be discovered (D. Johnson et al., 1986). All mature NTs bind to $p75^{NTR}$ with similar low affinity (MV, 2003). However, human keratinocytes have more than 200000 binding sites per cell for $p75^{NTR}$ as compared to approximately 1000 Trk receptor binding sites (Di Marco et al., 1993). The biological activity of $p75^{NTR}$ is twofold: first,

$p75^{NTR}$ is a Trk co-receptor that enhances NT-mediated Trk receptor activity and suppresses $p75^{NTR}$ induced death signaling (Di Marco et al., 1995).Second, in the absence of Trk receptors $p75^{NTR}$ autonomously or in conjunction with non- Trk co-receptors such as sortilin receptor complex activates signaling cascades that mostly result in the induction of apoptosis. Sortilin thereby binds specifically the pro-domain of NGF or BNDF (Lu et al., 2005). These cascades involve adaptor molecules such as the MAGE gene family and c-Jun N-terminal kinase (JNK) (Botchkarev, Yaar, Peters, et al., 2006). However, under certain conditions sole $p75^{NTR}$ signaling may also promote survival initiated by nuclear factor κB (NFκB) (Botchkarev, Yaar, Peters, et al., 2006).

The 149kD Trk receptor subfamily members bind their individual ligands with high affinity. TrkA is specific for NGF, TrkB for BDNF and NT4/5, whereas TrkC preferentially binds NT-3(Dechant, 2001). Basically, the Trk receptors are responsible for most of the survival and growth properties of the NT family. Tyrosine phosphorylation of these receptors stimulates the activity of cellular proteins responsible for regulating the cell shape and for activating the gene transcriptional that controls the fate of neural precursors, axon and dendrite growth and patterning, including growth, migration and morphology of keratinocytes, immune cells and other skin related cell types. The NT signaling through Trks controls the expression and activity of functionally important proteins, such as ion channels and neurotransmitter receptors (Huang & Reichardt, 2003).

The differential functions of individual and combined NT receptors demonstrate that the promotion of cell survival versus cell death depends on ligand availability prior to receptor and downstream signaling. Processing of proneurotrophins requires activity of proteolytic enzymes such as furins and prohormone convertase. These factors are responsible intracellular sorting of NTs to constitutive or regulated secretory pathway. Moreover, plasmin and selective matrix metalloproteinases (Lee, Kemani, Teng, & Hemstead, 2001) are also capable to cleave proneurotrophins in the extracellular space. Apparently these enzymes are abundantly present in the skin.

Skin is a highly complex tissue located at the interface between self and environment that helps to protect us from heat and cold, defend against physical and microbial stressors, sense our surroundings and communicate our feelings to the world around us. To this end, nerve fibers connect to the brain, keratinocytes of the epidermis and hair follicle form a barrier, fibroblasts provide elasticity and structure, blood vessels regulate flushing, temperature and the influx of mobile cutaneous cell populations, and last but not least immunocytes defend against microbial and chemical intruders and keep up our physical integrity. All of these features can be modulated by NT signaling and the entire NT signaling machinery has been detected in skin (Botchkarev, Yaar, Peters, et al., 2006).

The NTs function in skin is regulation of cutaneous innervation (Levi-Montalcini & Cohen, 1960), with the nerve fibers in skin target-derived NTs are retrogradely transported to the dorsal root ganglia through a complex signaling pathway (Howe & Mobley, 2005) and support the survival of neurons (Hempstead, 2004). Davies et al. (1987) demonstrate in developing skin, NGF synthesis begins with sensory innervations, and sensory neurons do not express NGF receptors until their fibers reach cutaneous target. Thus, cutaneous NT expression depends on innervation and conversely, lack of target organ development and cutaneous hypertrophy in denervated skin.

Schicho, Skofitsch, and Donnerer (1999) proved that NT treatment was shown to markedly improve cutaneous innervation after denervation. Transgenic mouse models have shown overexpression of NGF in the skin is responsible for outgrowth, hypertrophy and occasionally aberrant innervation of sympathetic fibers (Rice et al., 1998). They also induce outgrowth and hypertrophy but not aberrant innervation of cutaneous Aδ and C- sensory axons, the sensory neuropeptide and stress-mediator substance P (Stucky et al., 1999). However, neuropeptide expression within these nerve fibers may be altered. NT-3 by contrast is more responsible for the formation of sensory endings than axonal outgrowth (Rice et al., 1998).

Merkel cells including its innervation are supported by NT-3 via TrkC (Sieber-Blum, Szeder, & Grim, 2004), and supported by BDNF (LeMaster et al., 1999). In addition, NT-3 and NT-4 are responsible for hair follicle innervation around the neck of the hair follicle, meanwhile, NGF and NT-3 are responsible for sensory circular and longitudinal hair follicle innervation and BDNF for longitudinal innervation around the stem cells containing isthmus and bulge region of the hair follicle (Stucky, Shin, & Lewin, 2002). Finally, BDNF is responsible for noradregenic innervation of the arrector pili muscle associated with the hair follicle (Botchkarev et al., 1998). However, NTs do not always signal individually or synergistically in nerve fiber growth and maintenance, where occasionally the actions of one NT are antagonized by another. These effects ensure spatio-temporal differentiation of specific nerve fiber. Among the NT, NGF, being the most potent and diverse member of the family, plays a central role, and its functions are highly regulated by other NTs signaling (Lee, Kemani, Teng, & Hemstead, 2001).

These results clearly demonstrate that NTs exert neurotrophic effects at the skin level. Thereby, they play an essential role in the development of neural crest-derived sympathetic and sensory neurons in skin and are crucial for correct and timely development of cutaneous innervation. During adult life, however, they are equally important in the maintenance, the survival, and the promotion of neurite outgrowth and somatic enlargement in skin (Snider, 1994), and regulate livelong neuronal plasticity and adaptation to endogenous and environmental challenges (Peters et al., 2004).

NGF is released in increasing amounts by proliferating keratinocytes, while secretion ends in more differentiated cells (Pincelli et al., 1994). Di Marco an co-workers (1991) demonstrated that normal human keratinocytes synthesize and secrete biologically active NGF. Exogenous and endogenous NGFs have the capability of inducing keratinocyte proliferation, by demonstrating a direct effect of NGF on 3H-thymidine incorporation and inhibition of keratinocyte growth (Di Marco et al., 1993). Autocrine growth promotion is facilitated by co-expression of TrkA and $p75^{NTR}$ in keratinocytes in the basal epidermis (TrkA is expressed at the basal epidermis (Pincelli & Marconi, 2000). In addition to high levels of NGF, keratinocytes express and release low levels of other members of NT family. With increase in concentrations of anti-NT-3 antibody it inhibits keratinocyte proliferation, indicating that autocrine NT-3 acts as a mitogen in human keratinocytes through the Trkc receptor (Marconi et al., 2003). Finally, the full-length TrkB could not be detected in human keratinocytes in agreement with the observation that BDNF and NT-4 do not stimulate human keratinocyte proliferation (Marconi et al., 2003). Truncated TrkB could be detected in human keratinocytes and may inhibit BDNF signaling. Interestingly, BDNF and NT-4 is known as "epitheliotrophins" in murine skin which contradict with human data (Botchkarev et al., 1999). These data show that keratinocyte NT signaling takes a central position in epithelial tissue homeostasis, and seem to indicate that $p75^{NTR}$ acts as a proapoptotic receptor in murine and human keratinocytes (Botchkareva, et al., 2006).

Fibroblasts express $p75^{NTR}$, TrkA and TrkB, while they produce all of the NTs and NT signaling induces fibroblast proliferation *in vitro* and *in vivo* (Micera et al., 2001). In addition to keratinocyte and immunocyte signaling, NTs from fibroblasts in the affected area (Hasan et al., 2000) and in the granulation tissue during a wound healing process (Matsuda et al., 1998) may promote wound healing and associated inflammation.

Neurotrophins also play a central role in vascular development, which also underscores the close interdependence of nerve fiber and blood vessel development. Some direct or indirect effects of nerve growth factor (NGF) on angiogenesis and production of NGF by human dermal microvascular cells have been reported (Calza et al., 2001) in new born rats treated for 8 to 21 days with 6-hydroxydopamine and NGF, neuronal hypertrophy and endothelial cell hyperplasia associated with stromal hypertrophy and increased vascular bed were observed, suggesting NGF in combination 6-hydroxydopamine might contribute to neogenesis (Calza et al., 2001). It is interesting to note that in addition to their role as growth regulatory factors, NTs are among the main players of neurogenic inflammation (Steinhoff et al., 2003). NGF can activate mast cells, the central players of neurogenic inflammation in skin and induce their degranulation, which subsequently induces the classical neurogenic inflammatory cascade including increased blood flow and plasma extravasation (Peters et al., 2006). This cascade of inert inflammatory responses plays an important role in tissue maintenance, tissue repair, and host defense (Weller et al., 2006), but its overactivation causing inflammatory disease and tissue destruction.

A close proximity of mast cells to nerve fibers was noted and confirmed by many groups interested in neuro-immune interaction (Steinhoff et al., 2003; Suzuki et al., 1999). Mast cells also produced and released NGF and contribute to nerve growth, differentiation and function by producing NT. Human and rat mast cells express NGF mRNA, with human mast cell line HMC-1 is able to express NGF, BDNF, and NT-3 (Leon et al., 1994). Histamine and tryptase release from mast cells can be measured by monitoring serum and urinary levels, thereby demonstrating systematic effects of cutaneous mast cell activation. Skin derived NTs can exert its systematic

effects via this pathway to a local inflammatory impulse (Costa et al., 1996). NGF is large and stable neurohormone, and it is thus conceivable, that skin or even mast cell derived NGF may have systematic effects. However, the definitive origin of elevated systematic NGF-levels in cutaneous inflammatory disease remains to be determined. Mast cells are responsive to NT stimulation through expression of the TrkA, Trkb and TrkC receptors. Moreover NGF enhances expression of additional pro-inflammatory mediators and its signaling is able to initiate a complex adaptive cutaneous inflammatory response (Steinhoff et al., 2003).

In vitro studies indicate that proinflammatory cytokines such as interleukin -1β (IL-1β), TNF-α, interferon- γ (IFN-γ) , or IL-6 are able to modulate expression of NT and their receptors in dorsal root ganglion neurons (Cafferty et al., 2004; Murphy et al., 2000), Schawnn cells (Lindholm, Heumann, Meyer, & Thoenen, 1987), peripheral blood monocytes (Schulte-Herbruggen et al., 2005), and fibroblasts (Gadient, Cron, & Otten, 1990). NT activation of structural cells may play role in brain-to-skin interaction and mediate potent activation and modulation of immune function in various skin related immunocytes. NGF, BDNF, NT-3, and their respective receptors are found on blood derived monocytes and are inducible by inflammatory challenges in these cells (Rost et al., 2005). Based from the above listed signaling pathways and cellular effects of neurotrophins it become evident, that NT signaling in skin can take two major roads, for tissue homeostasis and repair, and it acts destructive in a multitude of diseases.

Wounding and associated inflammatory processes enhance NGF production mainly from keratinocytes in the affected area (Hasan et al., 2000). Keratinocyte NGF production is highest at the edge of the wound (Matsuda et al., 1998). This wound healing effect is possible due to anti-inflammatory capacities of NGF in the context of acute injury, since suppression of toxic edema was described (Banks, Vernon, & Warner, 1984). Also, NGF may be responsible for a phenotypic switch of keratinocytes, which facilitates wound healing (Micera et al., 2006). Cutaneous wounds often cause various kinds of damage to peripheral sensory neurons in the skin and NGF is essential to regenerate the injured neurons. In addition, NGF acts as a strong chemotactic factor for neutrophils, macrophages, and mast cells (Sawada et al., 2000) and is thereby essential for the early inflammatory phase of wound healing. Since NGF production is enhanced by the stimulation with various proinflammatory cytokines including IL-1β (Tanaka et al., 2004), an important role of NGF in wound repair at the site of inflammation was identified (Matsuda et al., 1998).

An inflammatory diseases on skin and other epithelial organs such as atopic dermatitis, psoriasis, allergic asthma, nummular eczema, keratoconjunctivitis, and premature hair follicle regression (Jarvikallio, Harvima, & Naukkarinen, 2003; Peters et al., 2004), the stress response has been associated with increased serum/plasma levels of NT. Acute and chronic stressors such as intermale aggression, para- chute jumping, noise, anxiety, labor or care giving (Alleva & Santucci, 2001) can increase plasma and peripheral levels of NT such as NGF in healthy and atopic individuals. NT may thus be the agents of the frequently observed worsening of cutaneous inflammatory diseases through stress exposure (Wright, Cohen, & Cohen, 2005). In addition environmental challenges such as noise stress apparently induce local changes in NT expression

in skin. In a model for noise induced stress, the cutaneous expression of NGF is increased along with increased $p75^{NTR}$ expression and decreased TrkA expression, which lead to premature termination of hair growth and apoptosis of hair follicle keratinocytes (Peters et al., 2004). This NT stress-response could be counterbalanced by NGF neutralizing treatment.

Stress elevated NT levels may affect the local and systemic immune responses thereby NTs act as stress mediators, which affect inflammatory skin diseases via neuro-immunemodulation. Vice versa, the observed increase in NT plasma levels in many inflammatory skin diseases may reflect activation of stress-response pathways especially in chronic and physically devastating disease. Furthermore, there is an increasing body of data supporting the idea that antigen-presenting activity is influenced by stress. Langerhans cells from stress- exposed mice show morphological changes, a reduction in cell density, and decrease in intensity of MHC expression (Kawaguchi et al., 1997). Mechanisms by which stress interferes with antigen presenting function of dendritic cells still have to be elucidated, but a presumable role of NGF, as an important stress mediator.

The above compiled evidence for a relationship between cutaneous innervation, structural cells and immune cells in skin homeostasis and disease regulated by NT signaling sketches a scenario, where NGF and its family members occupy a central position. Besides the regulation of keratinocyte proliferation, keratinocyte NT signaling may affect cutaneous adaptation to environmental and inflammatory challenges on several levels by providing neurotophins for nerve fibers and immunocytes. By regulating and enhancing cutaneous innervation and

neuroimmune contacts they stabilize brain to skin communication and via these pathways facilitate adaption to altered demands by environmental challenges such as UV or stress or endogenous needs such as hair growth or inflammation. By regulating cutaneous trophic functions in the epidermis, hair follicle and dermis, they participate in tissue remodeling processes in adaptation to these demands for example during wound healing (Kawamoto & Matsuda, 2004). However, hyperactivity of this signaling system is detrimental to allergic and chronic inflammatory skin diseases. Accordingly, NGF has been used for the treatment of wounds and in particular of human skin ulcers (Kawamoto & Matsuda, 2004) and at the same time improves innervation of these sites (Donnerer, 2003).

CHAPTER 3: MATERIALS AND METHOD

3.1 Study design

This is an animal study research using formalin fixed paraffin embedded tissue samples obtained from Research and Diagnostic Laboratory which was stored at –20°C involving expanded and control group in Faculty of Dentistry, University of Malaya. This study was approved by the Postgraduate Research Fund by Coursework (PPPC) Faculty of Dentistry, University of Malaya (PPPC/C1-2016/DGD/08).

3.2 Materials

3.2.1 Sample size calculation

Sample size estimation was done using G Power 3.1.9.4 for Mac OS X and sample size calculation based on previous study of (Kroeber et al., 2001), with power of 80% and alpha 0.05 for each center, the sample size suggested is a minimum of 10 animals per group.

3.2.2 Animals model

Institutional Review Board's approval was obtained before the commencement of this study (2015-161006/DENT/R/ZR). Animal model selection and management, surgical protocol and preparation were approved by the Animal Care and Use Committee, University of Malaya Medical Centre, Kuala Lumpur, Malaysia. Twenty female Sprague Dawley rats, aged eight weeks and above and each weighing about 180 – 200 g were equally divided into two groups of expanded (n= 10) and control (n= 10). The rat model has been used by many investigators prior for nerve expansion (1) and nerve regeneration (2). The capacity for regeneration is reported to be equivalent in rats and subhuman primates (Dolenšek, Rupnik, & Stožer, 2015). In this study, adult rats were chosen as an animal model as they are sufficiently large to allow surgical implantation of the expander within the scalp (Figure 3.1). In addition, adult rats were chosen in order to reduce the confounding factor attributed to growth. Skin tissues were harvested from ten female rats will be used as controls for tissue expansion, while ten female rats will be used to study in vivo tissue expansion. All animals were under the supervision of a Veterinary team (Laboratory Animal Centre, Faculty of Medicine, University of Malaya) and were treated according to humane guidelines.

Figure 3.1: Photograph of rat model after tissue expander implantation.

Figure 3.2: Photograph of rat model with expander in-situ in 12 days.

3.3 Selection of biomarkers

The selection of 2 proteins was obtained from (Wang et al., 2006) and (Shibayama & Koizumi, 1996): namely nestin and TrkB. These 2 markers previously have shown significant association and could predict the presence neurogenesis with high sensitivity and specificity.

3.4 Methods (Laboratory procedures)

3.4.1 Expander implantation

All surgical procedures were performed by the previous research group and supervised by the Principle Investigator (ZR). The procedure was done under general anesthesia (50mg/kg ketamine) and local infiltration (5mg/kg xylazine) in order to improve post-operative analgesia and minimize bleeding. Following the surgical procedure, the hair of the scalp region at the incision site was removed and permanent skin markings was tattooed using indelible ink. A sterilized anisotropic controlled rate self- inflating tissue expanders (Oxtex Ltd., Oxford, UK) were implanted beneath the subcutaneous layer of the scalp. The expanders were manufactured specifically for this study and designed to expand at a controlled rate in unidirection over 28 days. The surgical field was thoroughly scrubbed with 0.015% chlorhexidine gluconate and 0.15% cetrimide solutions (Baxter Healthcare Ltd., UK). A subcutaneous pocket was created by blunt dissection in a tension- free manner 5mm away from the incision site. The expander was slid through and implanted within the preformed pocket. The incision made during surgery was sutured and dressing was applied to allow healing. The rats were monitored and the rate of device swelling was determined by regular visual inspection and simple measurement using Vernier calipers (Mitutoyo, Japan) on a daily basis for 4 weeks prior to euthanasia (Figure 3.2). General condition of the subjects were monitored throughout the study.

3.4.2 Expander explantation and sample collection.

After the respective expansion periods were complete, euthanasia was carried out to remove the expander using carbon dioxide (CO_2) chamber. The expanded skin was surgically removed, snap frozen and stored at -20°C for further investigation. Control samples were collected from the same region as expanded samples from different rat which were not subjected to tissue expansion.

3.4.3 Specimen processing

Twenty samples of formalin fixed paraffin embedded female Sprague -Dawley tissues from the scalp region were collected. All tissue specimens had been fixed in 10% buffered formalin and embedded in paraffin blocks. Three sections with 5-7 μm thickness from the FFPE blocks and mounted on positive charged slides (Thermo Scientific, U.S.A). One of tissue section from each sample was stained with Haematoxylin & Eosin (H&E) (Sigma- Aldrich, U.S.A) and two other sections were used for immunohistochemistry (IHC) of nestin ((Rat- 401) Mouse mAb (CST)) and Trkb (NT-3, (CST)).

3.4.4 Tissue staining

3.4.4.1 Haematoxylin and Eosin (H&E) stain

Haematoxylin and Eosin (H&E) (Sigma- Aldrich, U.S.A) staining were applied to one sections of 5-7μm thickness from the scalp tissue of each groups for correlation of their histological diagnosis. Then, the sections were assessed for suitability for IHC staining by assessing the presence of adequate tissue representing the new nerve formation from each groups. The H&E staining method use is described in Appendix A.

3.4.4.2 Immunohistochemistry (IHC) stain

IHC procedures were carried out on all 20 representative control and expanded samples. Immunohistochemical procedures, incubation period, heat-induced epitope retrieval for antigen retrieval procedure, wash buffer, pH and temperature were employed for the primary antibodies based on the standard operating procedures based on literature and recommended based on manufacturer's data (Figure 3.3). Immunohistochemistry was performed using the Dakocytomation REAL EnVision Detection System-HRP according to manufacturer's specifications. The standard operating procedure of IHC staining for all markers is shown in Table 3.1. The IHC protocol use is described in Appendix B and C.

Gene symbol	Protein name	Source	Clone	Dilution	Antigen Retrieval Method	Incubation
Nestin	Nestin (Rat-401)Mouse mAb	E15 rat Spinal cord	SignalStain® Boost IHC Detection reagent (HRP Mouse)	1:200	HIER 0.01M citrate buffer pH6	Overnight at 4°C

TrkB	BDNF/ NT-3	Synthetic peptide	Anti- Rabbit IgG, HRP-linked Ab	1:50	HIER 0.01M citrate buffer pH6	Overnight at 4°C

Table 3.1: Immunohistochemistry Protein markers.

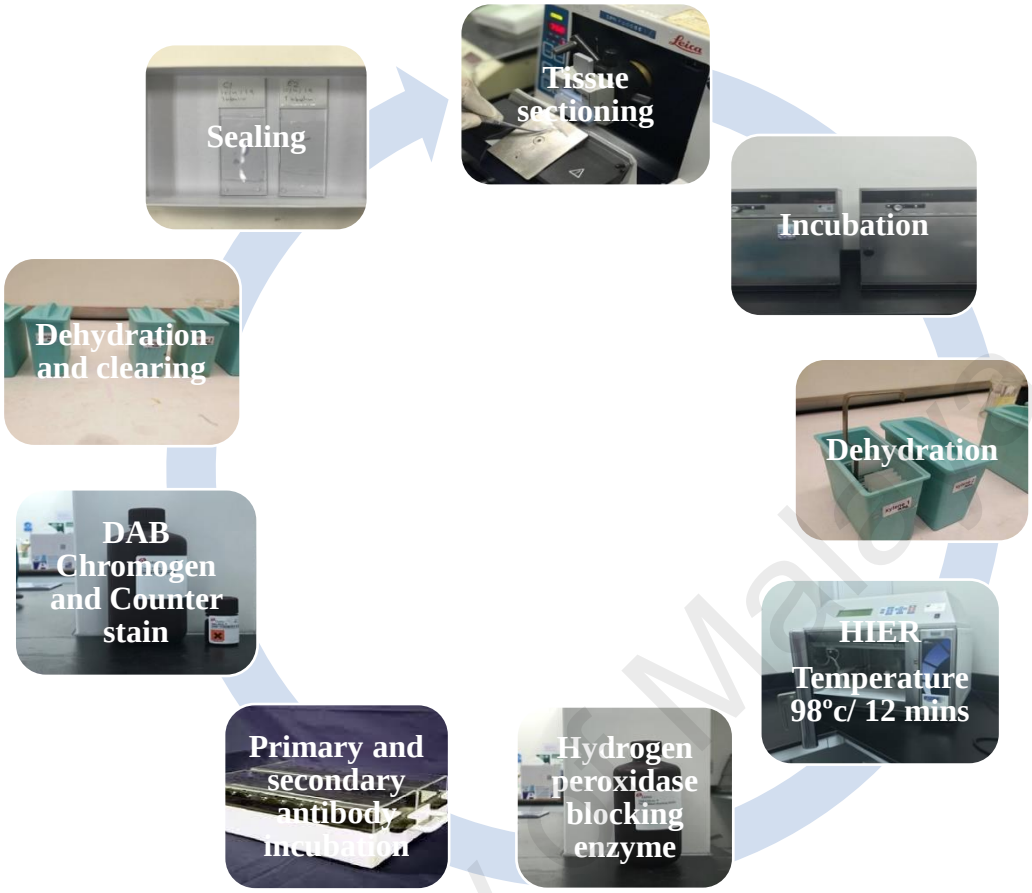

Figure 3.3: Photograph of Steps in Immunohistochemical staining.

3.5 Analysis

3.5.1 Calibration

Two assessors (oral medical pathologist and one orthodontist trainee) carried out the training and calibration session to identify the histological section of the samples. The assessors AKM (Medical Pathologist) and AJ (Investigator) were blinded to the control and expanded groups. Calibration exercise between the two assessors was held to evaluate the inter-observer agreement. Intraclass Correlation Coefficient (ICC) on SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) was used for assessing the inter-observer agreement level between the two assessors.

Table 3.2: Guidelines for interpretation of ICC inter-rater agreements (Cicchetti, 1994)

Score	Description
Less than 0.40	Poor
Between 0.40 and 0.59	Fair
Between 0.60 and 0.74	Good
Between 0.75 and 1.00	Excellent

3.5.2 Immunohistochemistry staining detection

The biomarkers were expressed in the cytoplasm and cell membranes of each tissue.

Table 3.3: Nestin and TrkB immunostaining on neural tissues.

Protein Markers	Subcellular location
Nestin	Cytoplasm, cell membrane
TrkB	Cytoplasm, cell membrane

3.6 Statistical analysis

Distribution of the data was analyzed using the Shapiro-Wilk. For comparison of independent variables of histological differences between expanded and normal skin samples, a parametric Independent T-test will be employed otherwise corresponding non parametric test, U-Mann Whitney test will be utilized. The data were considered statistically significant at $p < 0.05$. Data will be analysed using (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.). The result of protein expression will be reported as a descriptive data. Numerical results were presented as comparison of mean $\pm$ standard deviation.

CHAPTER 4: RESULTS

4.1 Sample Collection

The animal did not show obvious signs of distress or systematic illness throughout the study period. Skin infection, dehiscence, ulceration, or other adverse effects were not observed in any of the Sprague-Dawley rats. No substantial changes in body weight were observed between the experimental groups during the study periods. The soft tissue expanders were fully expanded in all experimental groups.

4.2 Histology

The Haematoxylin and Eosin stained sections showed twenty skins samples of expanded and non-expanded tissues with distinct layers consist of epidermis, dermis and hypodermis (Figure 4.1). Various morphology of keratinocytes are observed within the outer layer of skin in expanded and control group. The keratinocytes are aligned perpendicular to the underlying basement membrane to which they were anchored by special attachment structure (Figure 4.2). Increased mitotic activity was expressed by hyperkeratosis. Melanocytic activity was comparable in both groups (Figure 4.3).

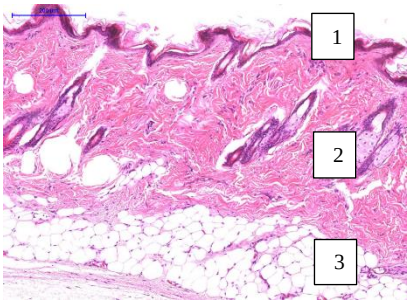 **A. CONTROL GROUP (CG)**	1.Epidermis layer - Pronounced rete ridge in CG compared to EG 2.Dermis layer - Collagen fibers are twinned and wavy in CG - Collagen fibers were parallel and stretch in the direction of stretching force in EG 3.Subcutanous layer - increased thicknes of the adipose layer in CG	 **B. EXPANDED GROUP (EG)**

Figure 4.1: Photomicrograph of different skin layer, consist of dermis, epidermis and subcutaneous layer (Magnification x10).

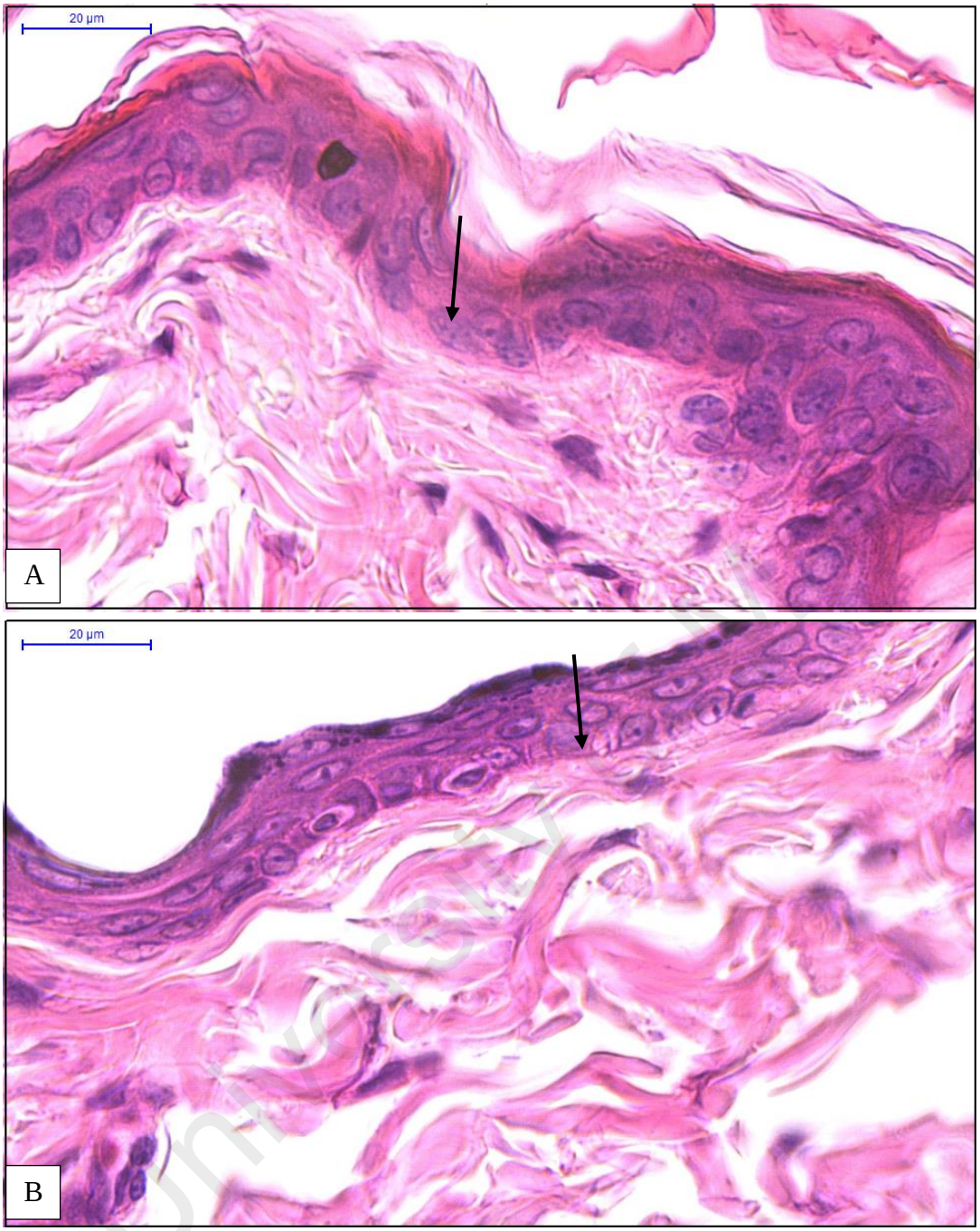

Figure 4.2: Photomicrograph show arrangement of keratinocytes (arrow) in normal skin (A) and expanded skin (B). The arrangement of keratinocytes in normal skin is perpendicular to the underlying basement, while in expanded skin, the arrangement of keratinocytes more parallel to the basement membrane (Magnification x80).

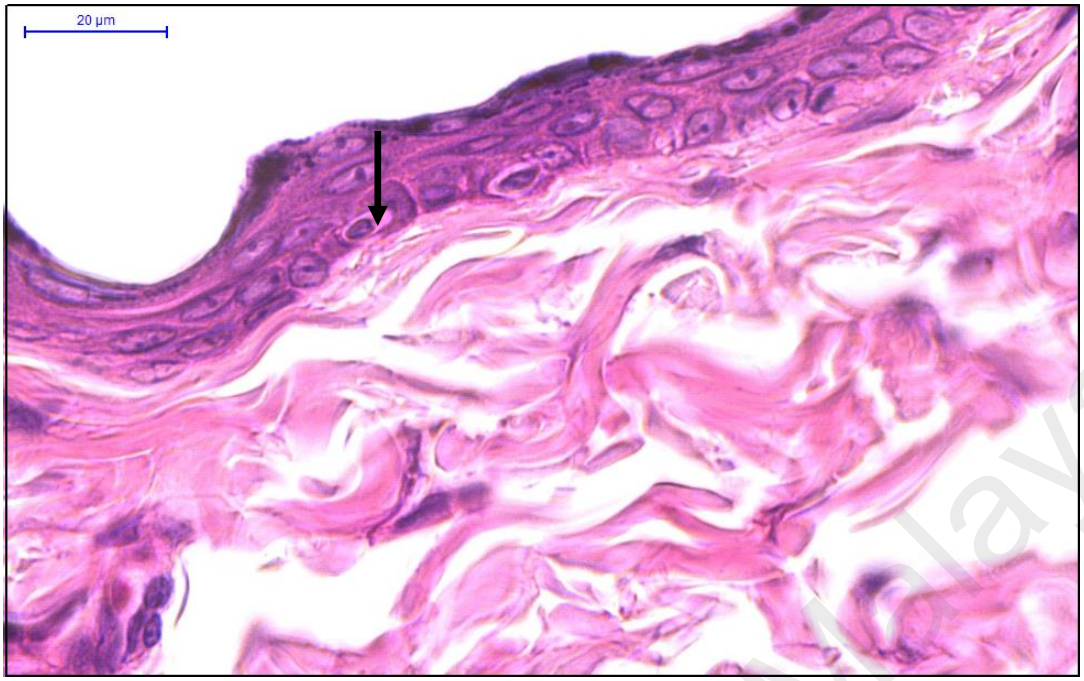

Figure 4.3: Photomicrograph of melanocytes in expanded skin (arrow) (Magnification x80).

In the epidermis layer, the cytoplasm of the basal epidermal cells and the prickle cells contained larger groups of tonofilaments scattered throughout the cells. The intercellular spaces in all layers of expanded epidermis were reduced compared to normal dermis. A few nonkeratinocytes in both groups were seen in comparable numbers (Langerhans cells) in the basal layer. The rete ridges more prominent in control skin compared to expanded skin (Figure 4.4). The basal lamina and basal surfaces of the basal epidermal cells demonstrated more undulation than the control groups.

The expanded dermis, especially the reticular layer, contained large bundles of compacted collagen fibers demonstrating the normal cross-banded. Fibroblasts were found much more frequently than in normal dermis and showed more active cisternae of the rough endoplasmic reticulum. A few myofibroblasts were seen in the deeper part of the dermis. A basal lamina-like material also surrounded some parts of the myofibroblasts. Skin appendages, capillaries, and small blood vessels showed no alterations. No inflammatory cells were found in the dermis in any of the expanded tissue.

A decrease in muscle thickness and mass occurs during expansion but muscle function is maintained (Figure 4.4). The myofibrils are enlarged and the number and size of mitochondria are increased. The sarcomeres are arranged aberrantly and contain irregularly scattered myofilaments. Within the subcutaneous tissue contained a paucity of adipose tissue and thickened collagen fibers in the interlobular spaces. The blood vessels of the subcutaneous tissue showed no changes.

A dense fibrous capsule develops around tissue expanders, the capsule is thickest and composed of elongated fibroblasts and a few myofibroblasts. These cells lie between thick collagen bundles oriented parallel to the surface of the implant. The fibroblasts contain very active rough endoplasmic reticulum with prominent cisternae, and many of these cells have numerous vesicles filled with dense material. Collagen fibers are found within the cytoplasm of the fibroblasts. The intercellular collagen fibers may be multiple. Most of the fibers are straight, some may be coiled or bent. The capsular membrane thins after removal of the tissue expander.

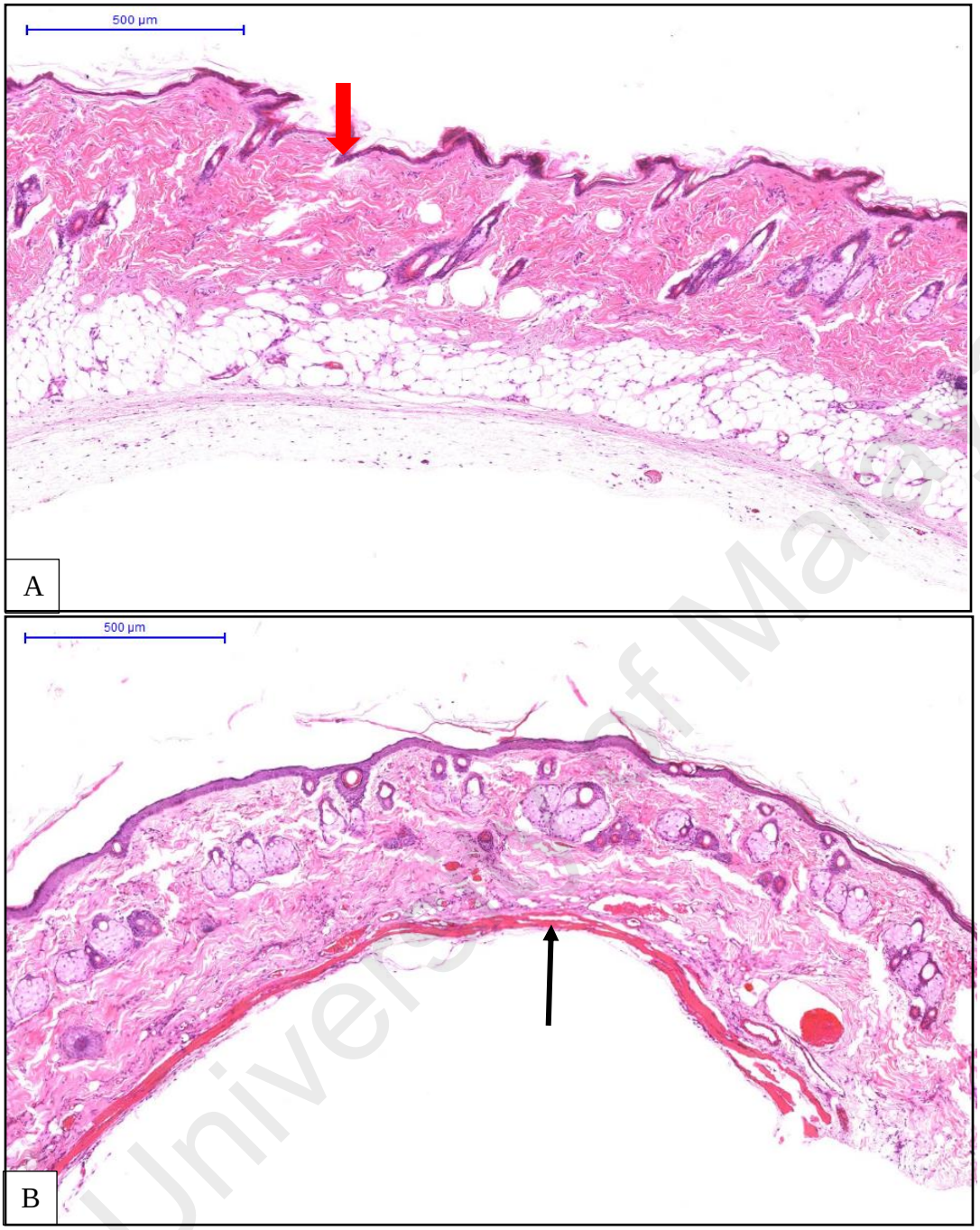

Figure 4.4: Photomicrograph showing absent rete ridges, with reduce muscle (arrow) thickness in expanded skin (B) comparing to normal skin (A) (pronounced rete ridges- red arrow) (Magnification x5).

4.3 Skin measurements

Skin expansion resulted in the significant increase ($p<0.05$) in thickness of epidermal layer and decrease ($p<0.05$) in thickness of the dermal layer in comparison with the samples obtained from control group (Figure 4.5). The epidermal thickness (μm) of normal skin samples (mean±SE [standard error]) (Table 4.1), from control group of rats were presented in a bar graph (Figure 4.6), respectively, compared to the expanded skin samples. For all groups, the increased epidermal thickness of the expanded skin specimens was statistically significant ($p<0.05$) from their corresponding control skin specimens.

The dermal thickness (μm) of normal and expanded skin samples (mean ± SE) of both groups (control and expande) was presented in the Table 4.4 including the bar graph in Figure 4.7. For all groups, the decreased epidermal thickness of the expanded skin specimens was statistically significant ($p<0.05$) compared with corresponding normal skin specimens.

The decreased in dermal thickness was compensated by the thickness of the fibrous capsule formed around the implant (Figure 4.8). Adnexal structures such as hair follicles and sebaceous glands became separated in the expanded skin, but they maintained their normal morphology (Figure 4.9). For all groups, the decreased number of the adnexal structures of the expanded skin specimens was statistically not significant compared to the normal specimens.

There was an increased blood supply in the expanded skin compared to the normal skin (Figure 4.10). It was shown by increase in size as well as the vascularity of the capsule surrounding the implant. The increased number of fibroblast and increased collagen deposition in the dermis were the major tissue response to expansion process in comparison with the normal skin tissue (Figure 4.11). Large bundle of compact collagen were observed in the expanded dermis. For all groups, the increased number of fibroblast of the expanded skin specimens was statistically significant ($p<0.05$) compared to corresponding normal (control) skin specimens.

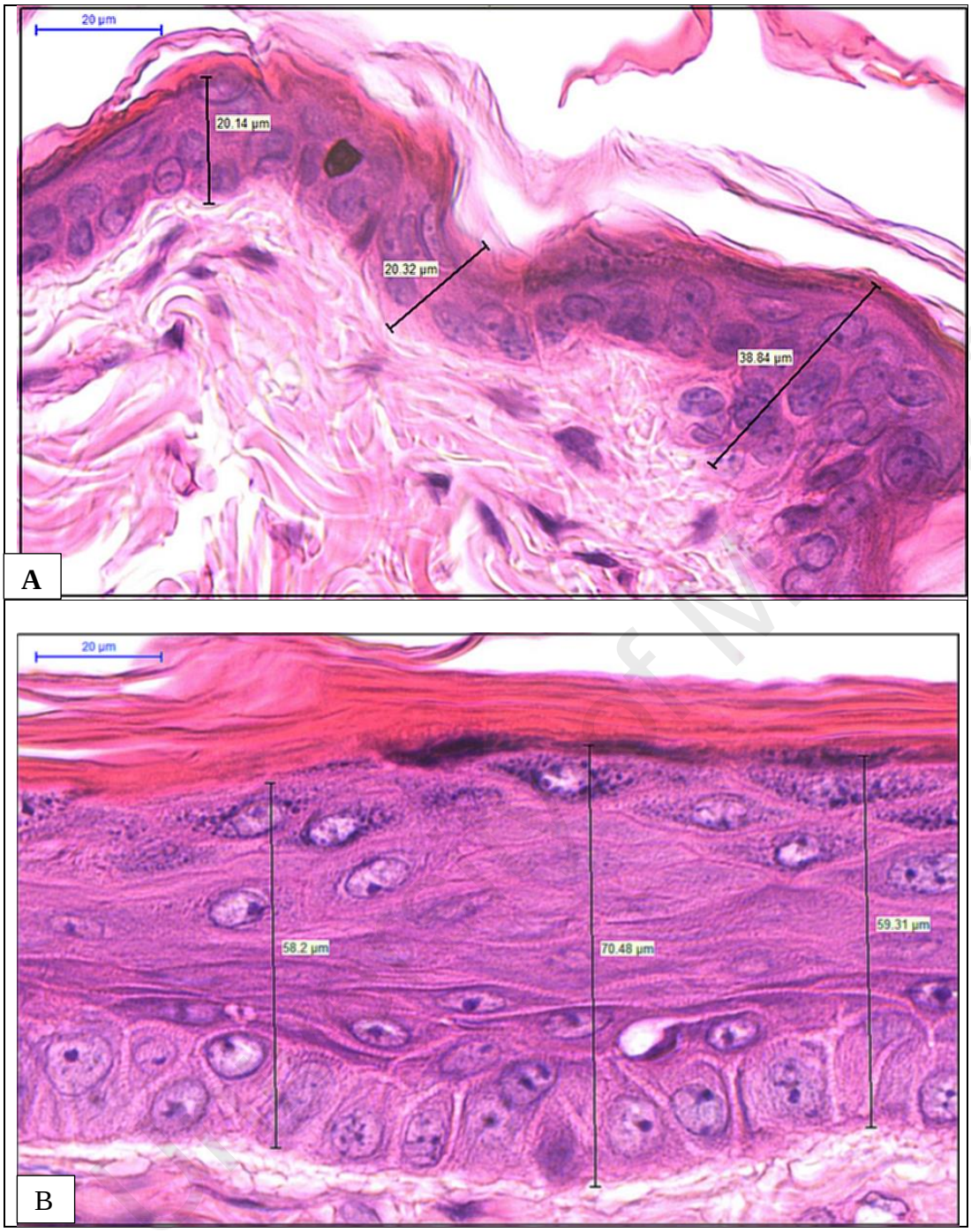

Figure 4.5: Photomicrograph of a histological section of normal skin tissue from neck area (A) and the same area that underwent skin expansion (B). Normal thickness of the epidermis and dermis layer in the normal skin and the expanded skin (Haematoxylin & Eosin stain; original magnification: 80x).

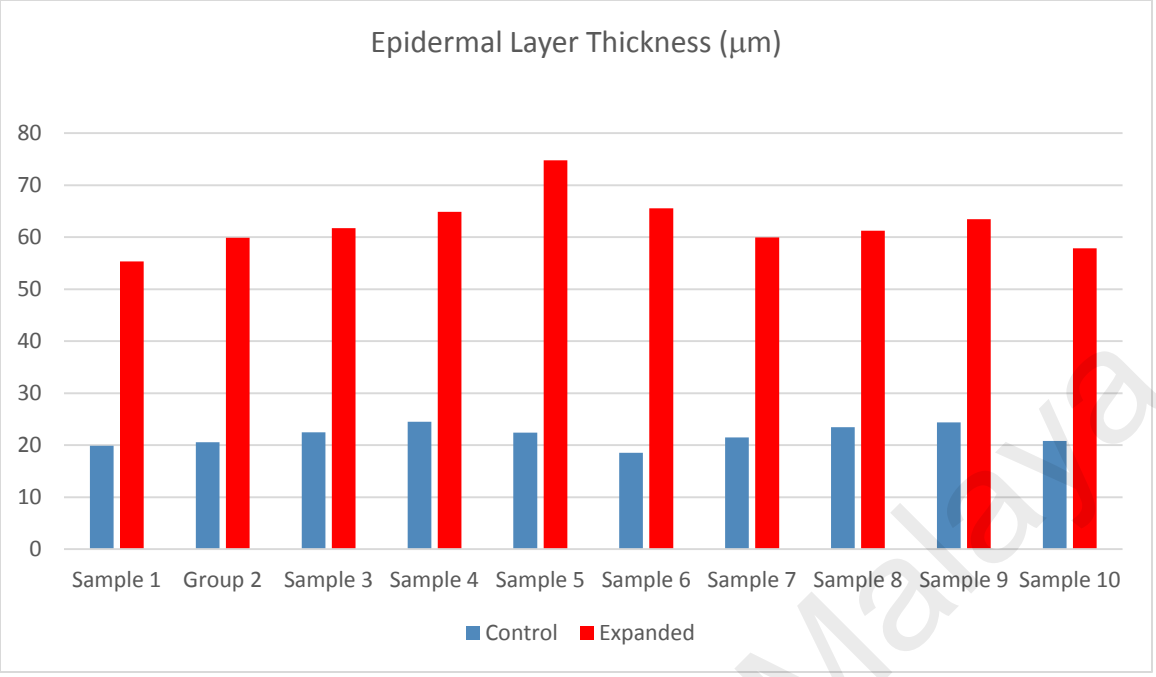

Figure 4.6: Epidermal layer thickness measurement between control and expanded skin.

Table 4.1: Mean ± SE for epidermal thickness between control and expanded sample.

Samples	Control	Expanded
Sample 1	19.88 ± 2.35	55.35 ± 5.45
Sample 2	20.56 ± 3.23	59.89 ± 4.15
Sample 3	22.45 ± 1.35	61.76 ± 3.59
Sample 4	24.5 ± 2.01	64.9 ± 3.02
Sample 5	22.4 ± 2.15	74.75 ± 1.35
Sample 6	18.55 ± 4.35	65.57 ± 3.01
Sample 7	21.5 ± 2.13	59.93 ± 4.02
Sample 8	23.45 ± 2.14	61.23 ± 3.50
Sample 9	24.35 ± 2.46	63.46 ± 3.12
Sample 10	20.78 ± 3.45	57.87 ± 4.46

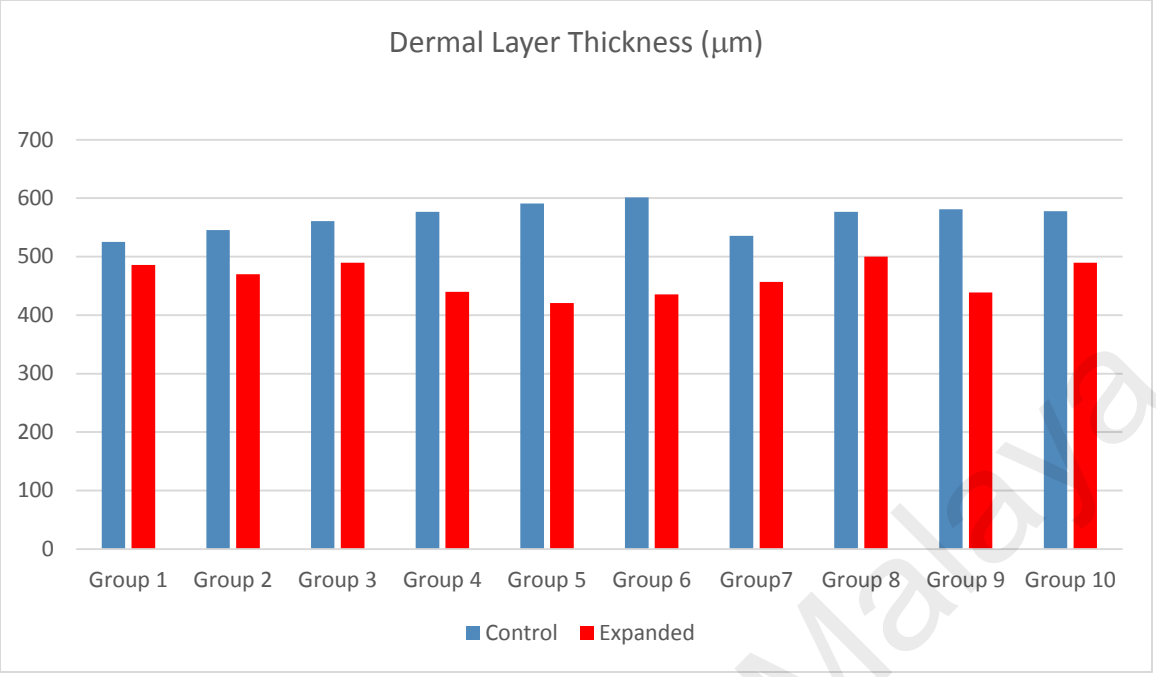

Figure 4.7: Dermal layer thickness measurement between control and expanded skin.

Table 4.2: Mean ± SE for epidermal thickness between control and expanded sample.

Samples	Control	Expanded
Sample 1	525.2 ± 9.35	485.7 ± 7.69
Sample 2	545.47 ± 8.95	469.89 ± 8.68
Sample 3	560.61 ± 6.79	489.47 ± 6.69
Sample 4	576.9 ± 5.78	439.89 ± 9.47
Sample 5	590.67 ± 4.59	420.45 ± 9.89
Sample 6	601.13 ± 3.98	435.44 ± 9.32
Sample 7	535.6 ± 8.99	456.65 ± 9.35
Sample 8	576.78 ± 5.57	499.89 ± 6.79
Sample 9	581.12 ± 5.23	438.7 ± 9.23
Sample 10	577.65 ± 5.67	489.39 ± 6.65

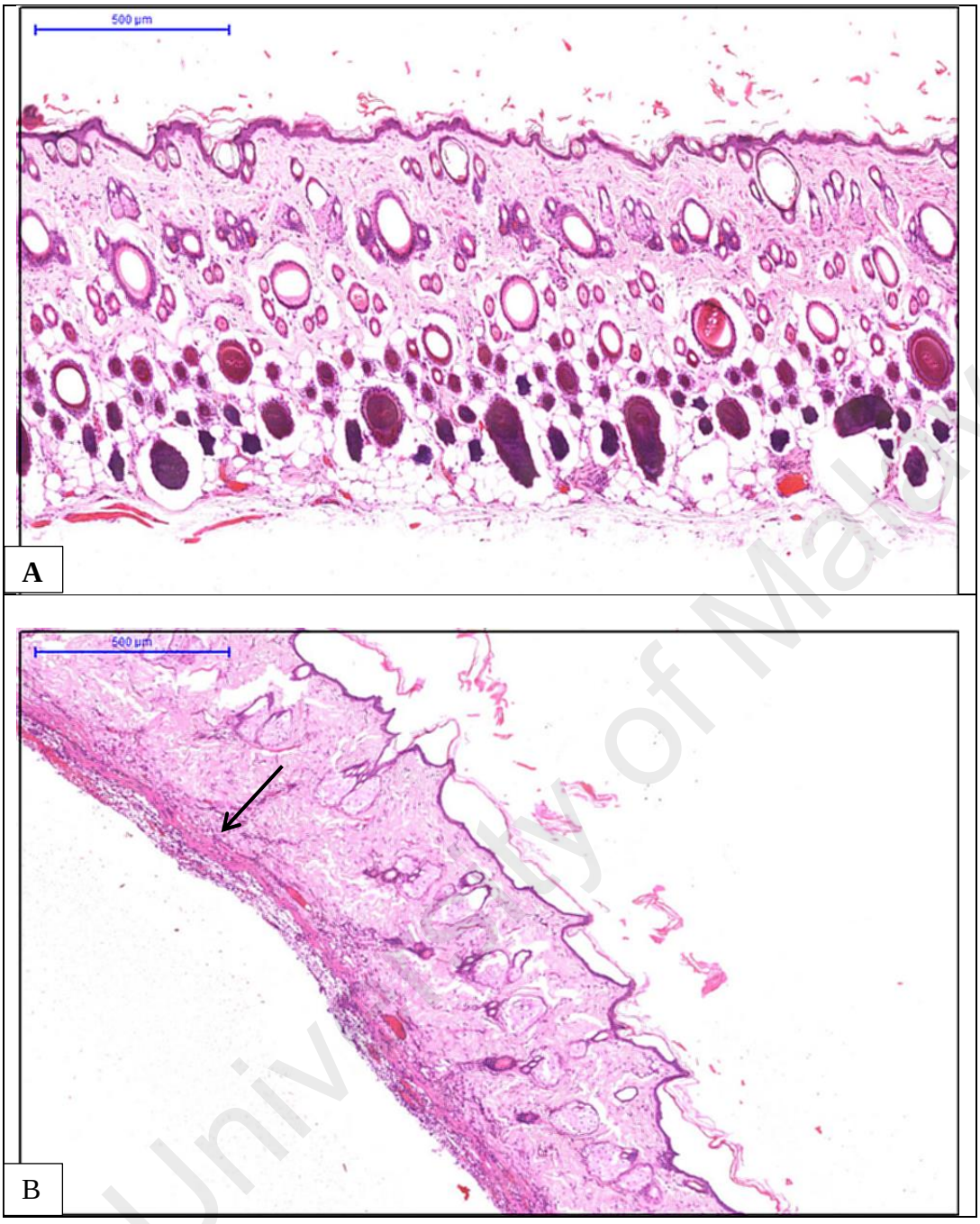

Figure 4.8: Photomicrograph of a histological section showing the capsule wall, with dense connective tissue, mild inflammatory reaction, and collagen fibers displayed parallel to the inner surface (B) (arrows pointed on the capsule wall). The delicate amorphous and eosinophilic material deposited on the inner edge (fibrin), normal skin (A) (Haematoxylin & Eosin stain; original magnification x5).

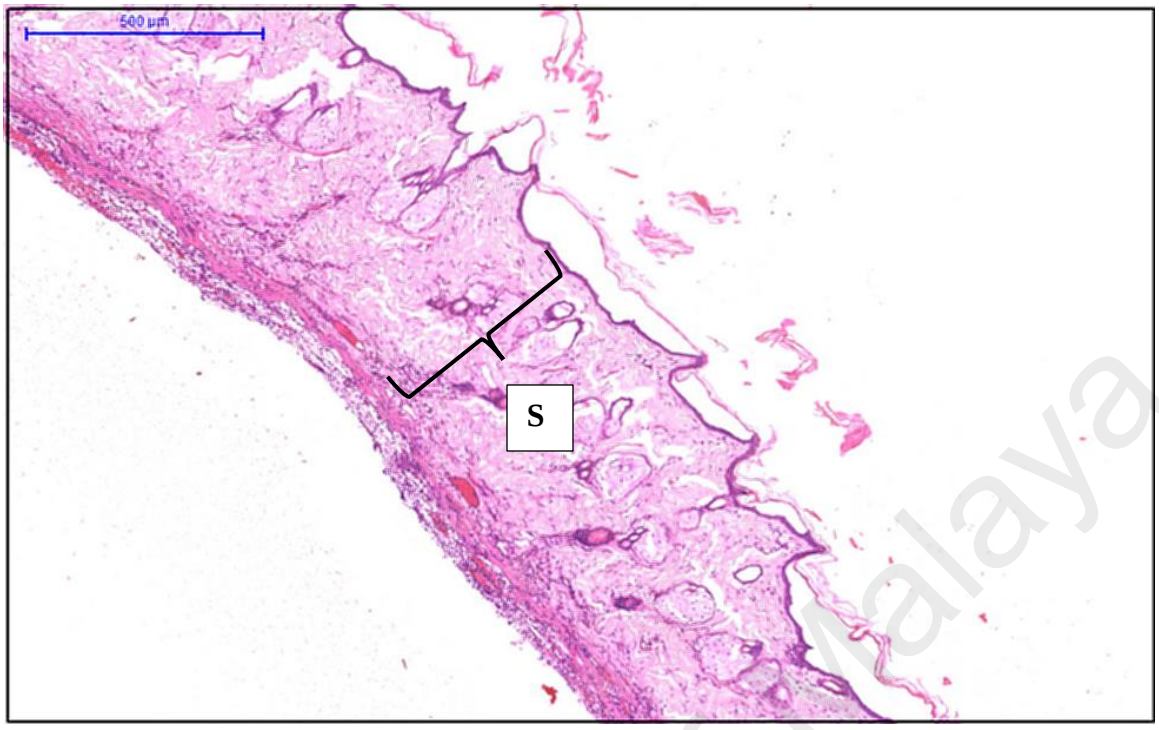

Figure 4.9: Photomicrograph of a histological section of expanded tissue with minimal adnexal structures ("**S**" indicating minimal adnexal structures in dermis region) (Original magnification x5).

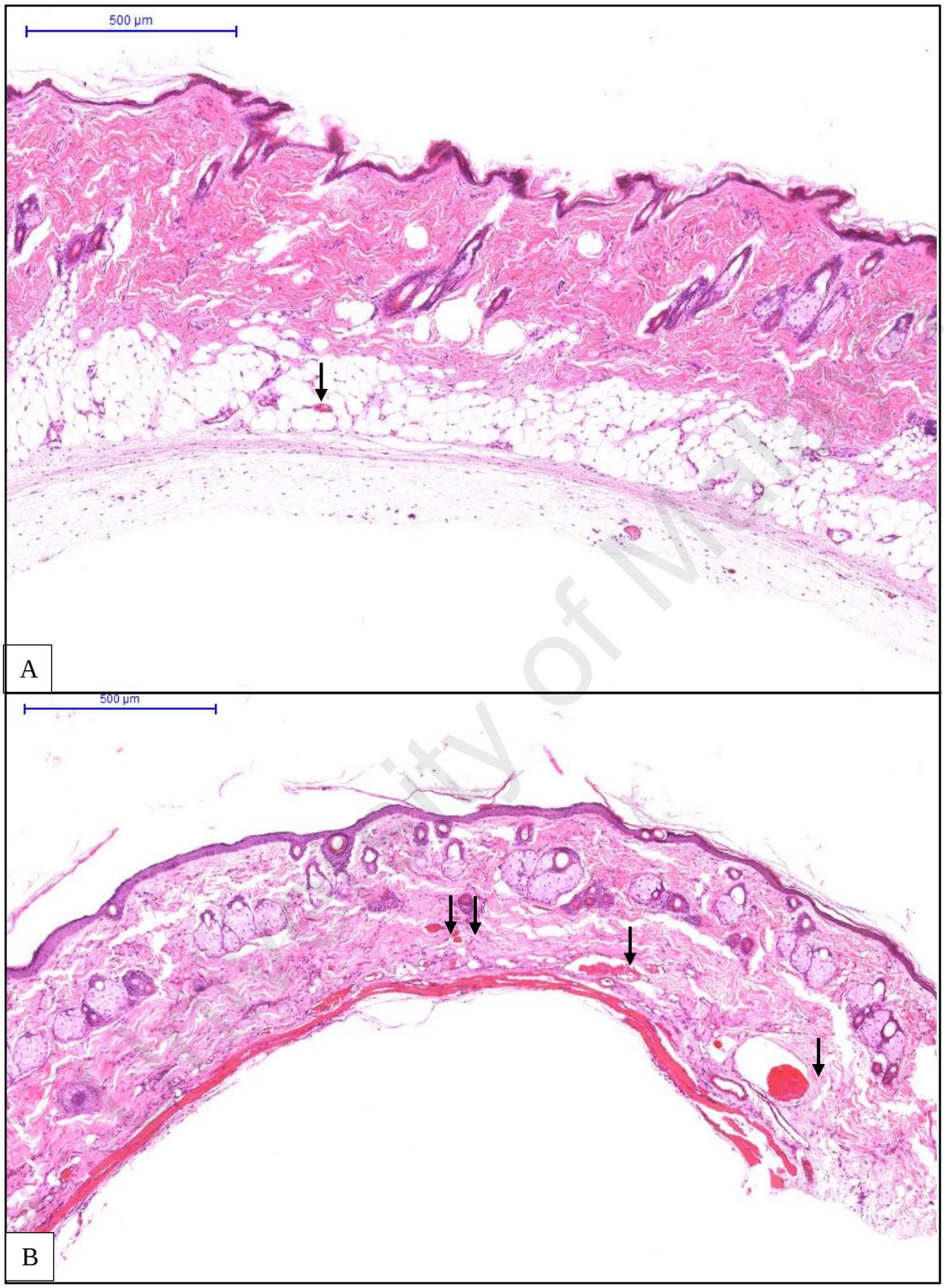

Figure 4.10: Photomicrograph of blood vessels in expanded skin (B) and normal skin (A) (Original Magnification x5)

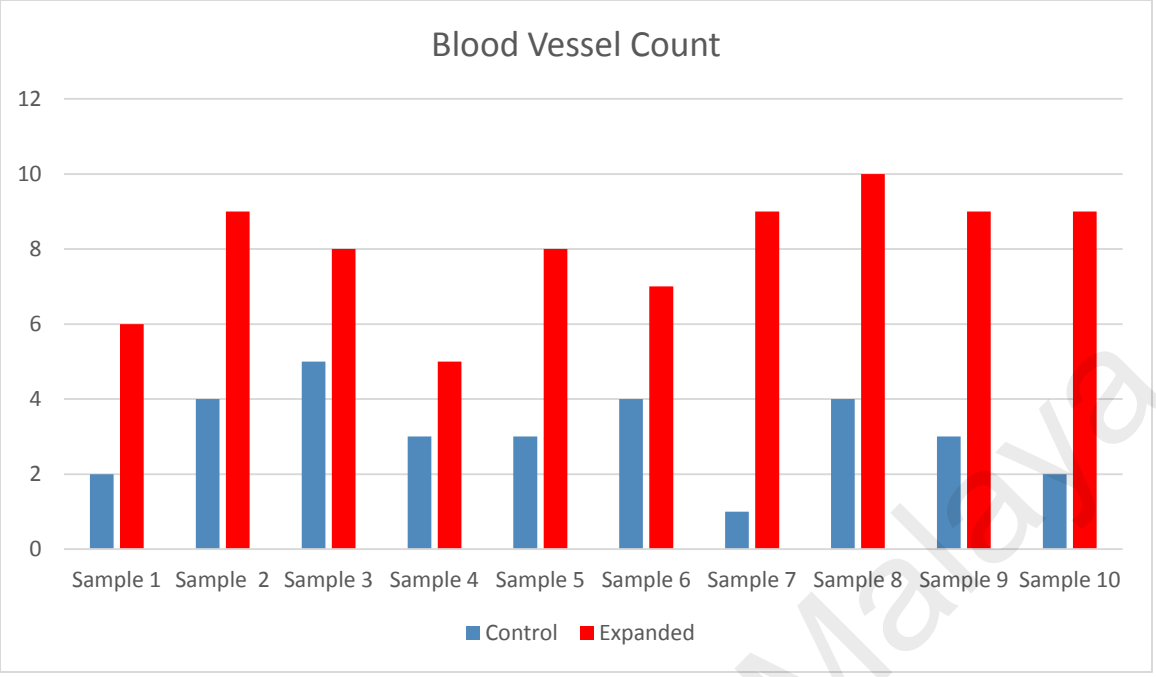

Figure 4.11: Number of blood vessel count increase in expanded skin compare to normal skin.

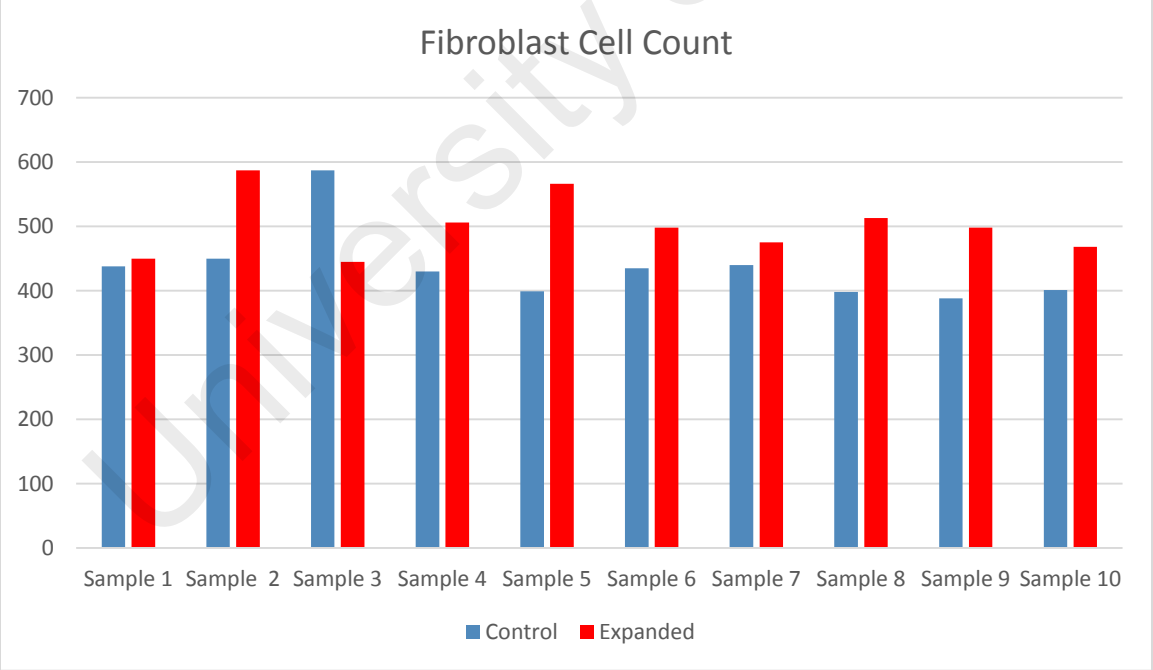

Figure 4.12: Increased number in fibroblast count for expanded skin compared to normal skin.

4.4 Descriptive Results

4.4.1 Immunohistochemical assessment

Overall, out of ten samples from expanded group only 30% were deemed as interpretable for analysis. The staining for nestin was localized in the cytoplasm and membranous protein of the skin cells. However, staining for Trkb protein markers not detected in any of the specimens.

4.4.2 Protein expression of nestin

In the non-expended skin, nestin was not detected or was detected in few dispersed cells in the epidermis, in the eccrine ducts and in hair follicles. In all expanded group, skin specimens from the expanded area showed intense expression of nestin in deep epidermal rete ridges. Strong immunohistochemical staining for nestin was observed in the epidermis; cells interspersed in the basal layer were more immunoreactive than those in the superbasal layer (Figure 4.12). Moreover, nestin-positive cells were detected in the outer root sheath basal layer of the hair follicle, but were not observed in the inner root sheath (Figure 4.14). Out of ten samples from expanded group only 30% expressed nestin expression. Strong nestin expression was detected in one on the expanded group with 95% of the skin section stained by nestin (Figure 4.13). In group 2 the nestin stained 95% of the sample but the intensity of the staining slightly faded compared to group 1. Meanwhile in group 3 the nestin expression mainly at the dermis area and can be found in the sebaceous gland, sweat gland and hair follicle (Figure 4.15). There was no significant difference in mean of nestin positive cell numbers between the study groups.

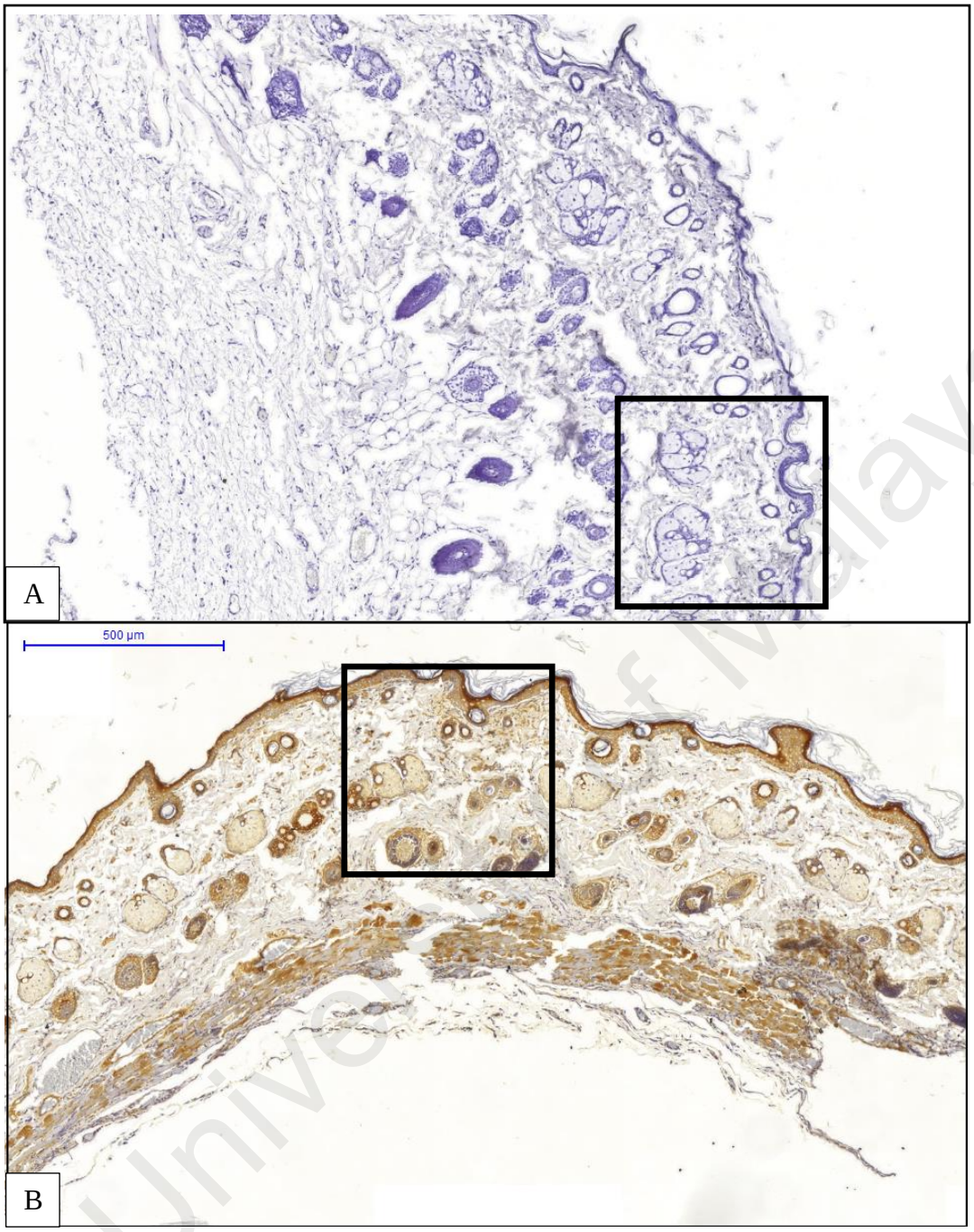

Figure 4.13: Photomicrograph of Nestin immunostaining showing strong staining intensity in the expanded skin (B) and no staining detected on normal skin (A) (Original magnification x5).

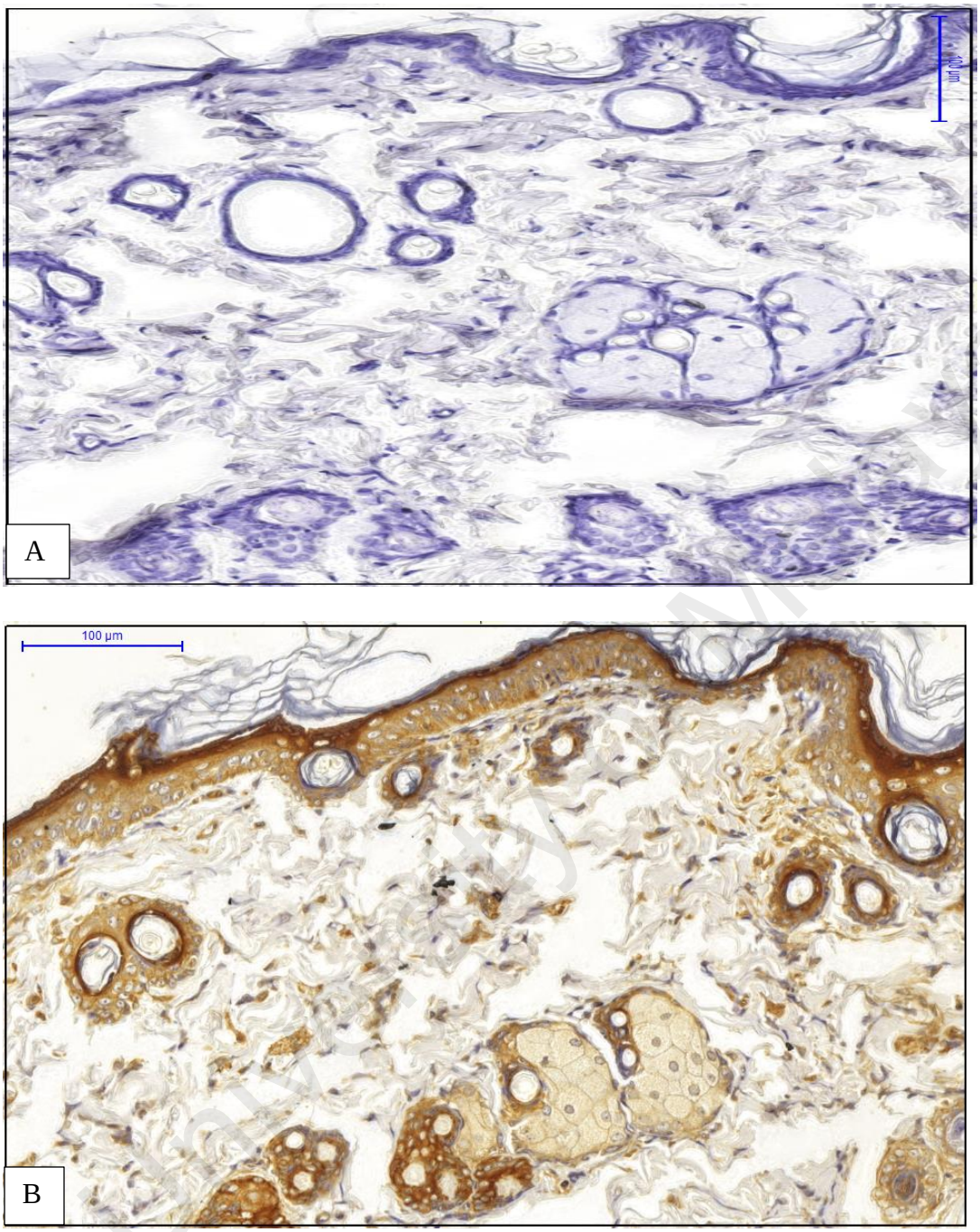

Figure 4.14: Photomicrograph of Nestin immunostaining showing strong staining intensity in the expanded skin (B) and no staining detected on normal skin (A) (Original magnification x20).

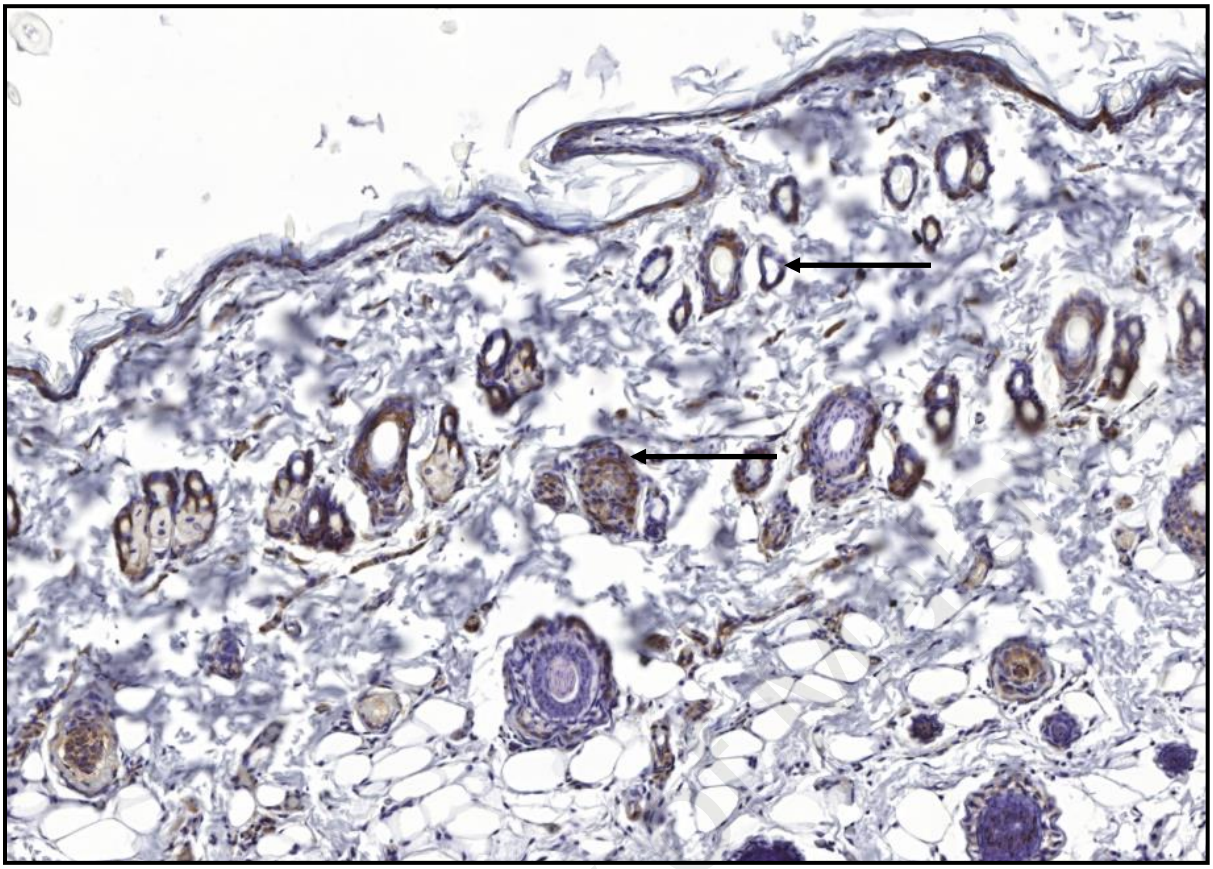

Figure 4.15: Photomicrograph of Nestin expression on the outer root sheath (Magnification x80).

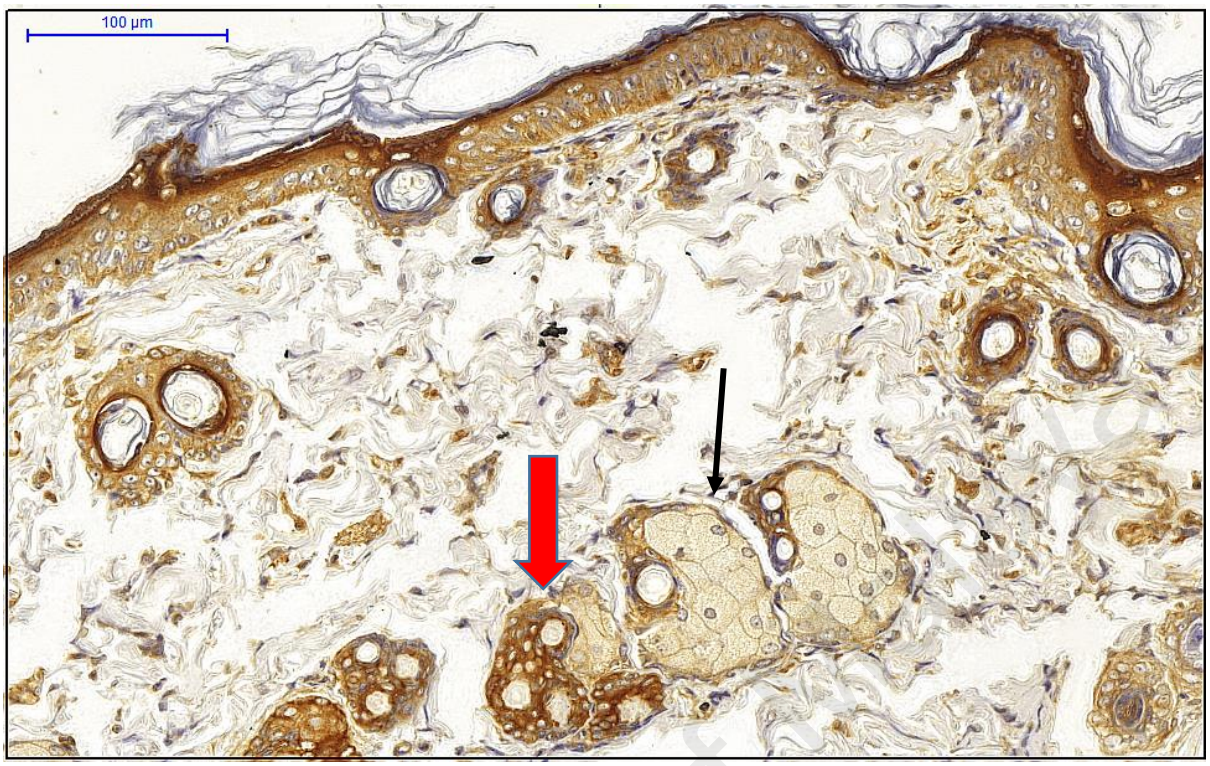

Figure 4.16: Photomicrograph of Nestin immunostaining sebaceous gland (black arrow) and sweat gland (red arrow) (Magnification x20).

4.4.3 Protein expression of TrkB.

All specimens from both tissue tested with Trkb protein markers, (expanded and control skin), revealed no levels of TrkB expression. Both groups shows a comparable results of TrkB expression (Figure 4.16).

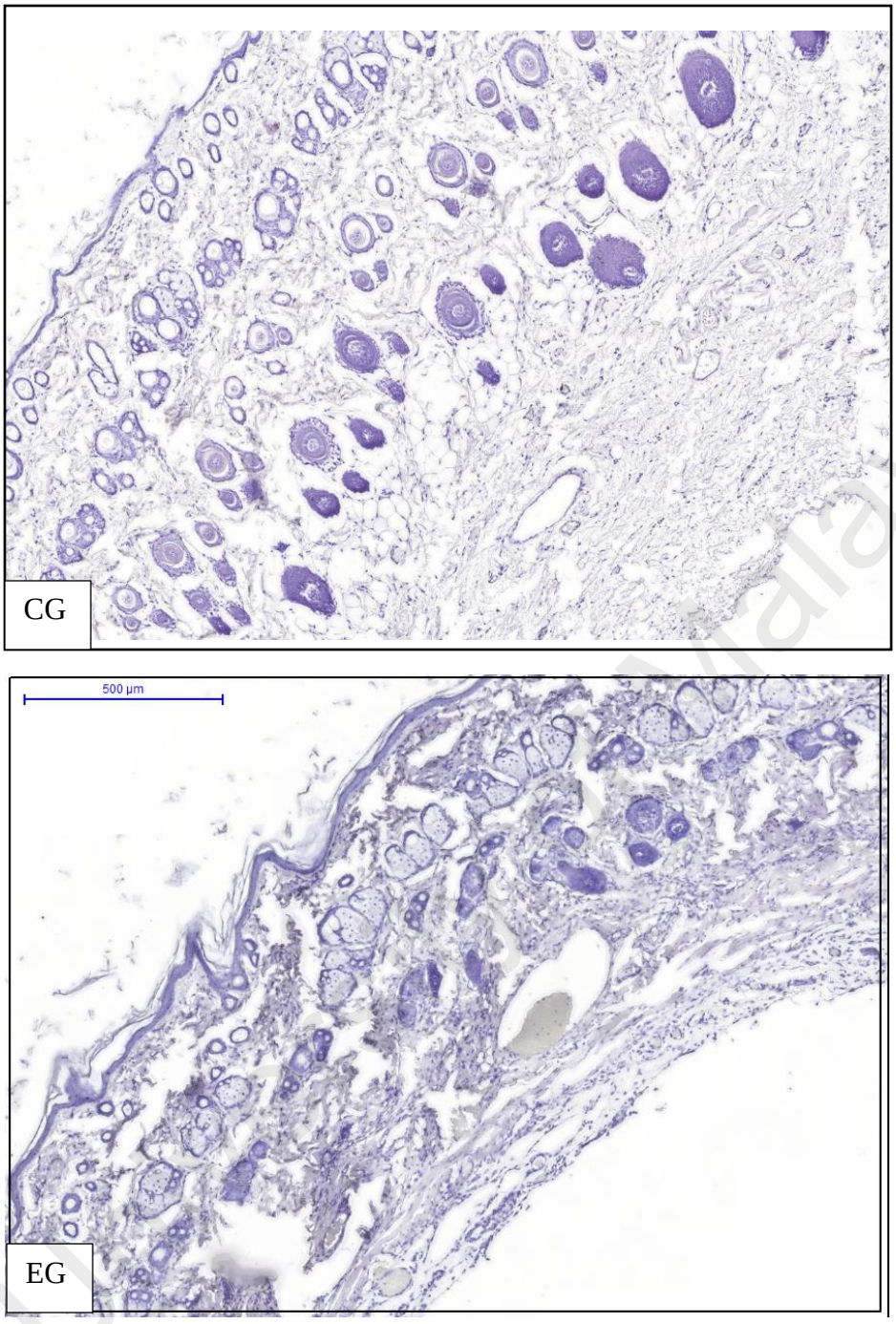

Figure 4.17: Photomicrograph of TrkB immunostaining on Control group (CG) and Expanded Group (EG) (Magnification x10).

CHAPTER 5: DISCUSSION

5.1 Animal selection

Selection of an appropriate animal model for tissue expansion is critical to allow the extrapolation of the findings to humans. Previous studies on tissue expander have been carried out on monkeys, rats, rabbits, cats, dogs, pig and sheep (Alyessary et al., 2017; Ali et al., 2018; Aziz et al., 2018; Wysocki et al., 2011; Uijlenbroek et al., 2011; Wollina et al., 1992). Moreover, the physiology is more like the corresponding human condition. The size of the animal enhances its use as a model, not just because of the ability to perform surgical procedures, but also because of the proportional size of important substructures to specific anatomical areas. This is particularly important in the neuronal research work. The rat model also more responsive in histopathology and resemble the human tissue.

5.2 Histology

This study demonstrates that in rat, an increased thickness of the epidermal layer and the decrease of the dermal layer thickness in response to tissue expansion. The significant decrease of dermal thickness could be directly implicated to stretching of the tissue and in agreement with the previous findings by Austad, Pasyk, Mcclatchey, and Cherry (1982), Austad and Rose (1982), Ali et al. (2018)and Tepole, Ploch, Wong, Gosain, and Kuhl (2011). On the other hand, the epidermal hyperkeratosis and acanthosis represented the increase in mitotic activity in the expanded skin. The epidermal mitotic activity is regulated by the activation of growth factor pathways which is triggered by epidermal tension (Razzak et al., 2016; Zöllner et al., 2013). This

mitotic activity regulated by cell density of the basal layer of the skin which is decreased by tissue expansion where greater cell proliferation is induced by lower cell density, leading to the growth of extra skin (Shan & Baker, 1991). According to Pasyk, Argenta, and Austad (1987) there was no correlation between thickness of the epidermis and time of expansion, size and volume of the inflated expander, location of insertion of the expander and age of the patient. The development of epidermal hyperkeratosis in expanded skin samples remarkable and coincide with previous findings (Johnson, Kernahan, & Bauer, 1988). There is a possibility due to the burst in epidermal proliferation (Austad, Thomas, & Pasyk, 1986) but another explanation could be due to the rubbing over the expanded sites. Such frictional stimulus might result in histochemical epidermal changes as well (Wollina et al., 1992).

In spite of some tension transfer to the epidermis, the significant thinning of the skin can be attributed to the maximum tension to which the dermis is exposed during expansion, leading to a significant thickening of the epidermis and thinning of both dermis and subcutaneous tissue (Johnson et al., 1993). This proved that the changes of epidermal and dermal layer thickness were one of the main macroscopic features of skin expansion in rats.

The results of the present study also showed an increased in melanocytic activity in the expanded skin. This possibility could be due to the clinical hyperpigmentation (Agrawal & Agrawal, 2012), as it was attributed to the hyperactivity of melanocytes during expansion and tends to be slowly reversible to normal in the event of expander or mechanical stimulus removal. The skin expansion also resulted in a significant decrease in the adnexal structures such as hair follicles and sebaceous glands, as they were found relatively farther apart and supported by other researchers (Argenta & Vanderkolk, 1987). At the same time the skin appendages (hair follicles, sweat and sebaceous glands) also showed some compression, but they did not demonstrate degeneration or histological changes during tissue expansion and this also coincides with findings by (Austad et al., 1986; Pasyk et al., 1987; van Rappard et al., 1988).

This experimental studies showed a statistically significant thinning of the dermis after tissue expansion and supported by others (Austad et al., 1982; Pasyk et al., 1982). However, the thinning of the dermis during expansion had no adverse effects on the blood supply (Cherry et al., 1983). Capillaries in the papillary dermis and the small vessels in the deep corium were dilated (Pasyk et al., 1987). Blood flow in the skin covering the expander however, was lower than in adjacent skin (Timmenga, Schoorl, Bos, & Klopper, 1989; Timmenga, Schoorl, & Klopper, 1990).

The result also showed the increased number of fibroblast (fibrodysplasia) and collagen deposition in the dermis in comparison with the normal skin. Large bundles of compacted collagen were observed in the expanded dermis. Tissue expansion resulted in an increased number of fibroblasts and the presence of compacted collagen and angiogenesis as reported in other study (Lantieri et al., 1998). The increase of collagen and extracellular matrix synthesized

by the fibroblast is a response to either the pressure by the tissue expander or traction of the fibroblast cytoskeleton by the extracellular matrix (Marcus et al., 1990). The mechanical load stimulated the extracellular matrix components to switch cells between growth, differentiation, and involution, whereas the synthesis and secretion of intracellular products are evoked within 24 h after a single mechanical stimulus. Large bundles of compacted collagen observed in the expanded dermis represented abundant quantities of immature collagen produced by the elevated numbers of fibroblasts within the dermis. These histological changes appeared to be one of the major tissue responses to expansion process and in this case for the creation of additional skin.

The increased vascularity of expanded skin was noticed in this study as compared to the normal skin. Previous study by (Leighton et al., 1988a; Leighton et al., 1988b). The capillaries not only increased but also dilated. The realignment of vessels, the neovascularization, and the depletion of neurohumoral vasoactive substances are the most factors seemed to be related to the explanation for increased vascularity in tissue expansion (von See et al., 2010). In this study, the implantation of the expander could lead to the formation of vascular fibrous capsule surrounding the implant as a result of tissue-foreign body reaction (Johnson et al., 1993). Therefore, the increased vascularity in expanded skin is due to tissue reaction involving the increase in the skin vascularity by having a circulation exceeding the subdermal plexus.

The effect of tissue expansion on peripheral nerves was studied by Milner (1989)The increase in length of the nerve was proportional to the degree of expansion. All nerves showed a decrease in conduction velocity. Normal conduction velocity after tissue expansion, however, was reported (Wood & Mcmahon, 1989). Endo and Nakayama (1993) reported over eight week period using tissue expander resulted in 8% average increase in length of the sciatic nerve. Yamada et al. (2009) also found that gradual lengthening of distal nerve stump possible to lengthen the nerve and increased the number of Schwann cells in the lengthening group. This proved that mechanical distraction could generate growth and new nerve.

5.3 Protein expression of Nestin.

In skin, each epithelium contains a hair follicle and its surrounding epidermis. The epidermis maintains its homeostasis by proliferating from the basal layer. As the basal cells detach from their underlying lamina they stop dividing, commit to terminal differentiation and eventually sloughed from the skin surface (Watt, 2002). An appendage of the mammalian epidermis, the hair follicle, is composed of the concentric rings of an external outer root sheath (ORS) attached to the basal lamina and contiguous with the epidermis, a channel (inner root sheath, IRS), and finally the hair shaft (Bernal & Arranz, 2018). Hair follicle cells with high proliferative potential reside in the bulge region of the upper portion of the follicle. In response to wounding, the bulge cells migrate and proliferate to repopulate a new follicle, and differentiate into dermis and sebaceous gland (Hoffman, 2006)

Nestin is an intermediate filament protein expressed in undifferentiated cells during central nervous system (CNS) development. It is specifically expressed in neuroepithelial stem cells and its expression distinguishes these cells from more differentiated cells in the neural tube, the property that inspired the name nestin (Lendahl, Zimmerman, & McKay, 1990). Recently, it has become clear that nestin is also expressed in other tissues and its down-regulated during tissue maturation. About and co-workers in 2000 reported that nestin is expressed in the mouse hair follicle cells located just above the bulge region and can differentiate into various cell types during wound healing (Houseweart & Cleveland, 1998) and can regulate the neovascularization of the dermis in association with the hair growth cycle (Wang et al., 2006).

The present results show that nestin is widely expressed in adult tissues (rat skin and its appendages). It is expressed in all layers of the epidermis, and in the cells interspersed in the basal layer; nestin staining here is darker than in the suprabasal layer. The immunohistochemical staining results show that nestin expression is stronger in the bulge region. After injury, it has been observed that cells re-express or upregulate nestin (Ernst & Christie, 2005) which may indicate extensive remodeling or a reversion to a more immature phenotype.

The molecular mechanisms of nestin reactivation after injury are complicated. Independent mechanisms, including extracellular factors, cell-cell interactions, transcriptional regulation and intermediate filament remodeling may be involve. Extra-cellular soluble factors may trigger gene activation after their release from the adjacent tissuses (Holmin, von Gertten, & Sandberg-Nordqvist, 2001). Up-regulation of nestin could be induced by changes in cell-cell contact patterns. (Parent, 2003). This findings suggest that complex gene regulatory events may be activated after cellular damage and regeneration. Similar findings found in early development, IF protein remodeling may be involved in regenerating cells after injury. In neuroepithelial cells of the neural tube, nestin plays a role in the organization and maintenance of the elongated cell morphologies (Gilyarov, 2008). Nestin also mediates phosphorylation-dependent disassembly of vimentin IF during mitosis in a concentration-dependent manner (Ernst & Christie, 2005).

All these supports our findings that nestin expression may be involved in tissue regeneration in adults. In addition to that, the tendency towards being present or regeneration of new nerve due to initial presence of nestin at the end of maximum expansion state on the expanded group.

5.4 Protein expression of TRKB

The result of this study could not detect the present of TrkB on rat skin as reported by others (Botchkarev et al., 1998; Di Marco et al., 1991). The previous study reported presence of NGF and NT-4 in basal epidermal keratinocytes in human and mice. (Botchkarev et al., 1998) also reported that BDNF and NT-3 are expressed in cutaneous nerve fibers and myocytes of the arrector pili and panniculus carnosus muscle. We speculate that the absence of TrkB in this study probably due to the condition of the samples and highly related to the human processing errors including sectioning the samples at 7μm thickness. According to (McCampbell et al., 2017) the use of tissue thickness outside vendors' recommendations might change the intensity including the proportion of positive and negative cells and eventually the overall diagnosis outcome. It is recommended that tissue be consistently cut within the middle thickness range specified by the assay manufacturer which is 5 μm. In order to overcome the increase of thickness, we optimized the primary incubation period exceeding overnight incubation in -4°C, but none of the samples show positive TrkB staining. The drastic changed in the sample thickness mainly due to the problems during heat-induced epitope retrieval process that causing the samples dislodged and burst after exposed to the heat for antigen retrieval procedure.

5.5 LIMITATIONS

The results of this animal study must be interpreted with caution when generalizing it to clinical application as there is species differences in term of anatomy, organ structure and function, toxin metabolism, chemical and drug absorption and mechanism of DNA repair of the tissue which can be quite different in human. In this study because of loss of biological variability or predictivity resulting from the use of in-bred strains, young animals, restriction to single sexes and inadequate group sizes contribute to limitation of the study. The physiological and immunological distortions resulting from stressful environments and procedures also contribute to the limitations of the study.

Most of the laboratory animals spend their lives in small, relatively barren cages, but the environments fail to ameliorate most of the deficits. Common laboratory species suffer marked stress, fear and possibly distress when subjected to handling, blood sampling and gavaging. Animals do not readily habituate to these procedures overtime. In addition, the stressful alteration of normal physiological parameter also predisposes to a range of pathologies and distorts the scientific results.

The false positive results in rat studies may be due to overwhelming of natural physiological defenses such as epithelial shedding, inducible enzymes, DNA and tissue repair mechanisms, which effectively protect against many naturally occurring toxins at environmentally relevant levels. Unnatural elevation of cell division rates during ad libitum feeding studies , plus, stresses caused by handling and restraint, and frequently stressful routes of administration, and subsequent effects on hormonal regulation, immune status and disease predisposition

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Within the limitation of the present study, the followings can be concluded:

1. The macroscopic changes in rats expanded skin showed that the tissue was able to rapidly adjust and compensate against mechanical stretching.
2. Increased epidermal thickness, reduced dermal thickness, an elevated number of fibroblasts, increased vascularity, and parallel orientation of collagen fibers were the main histological changes observed in response to tissue expansion.

3. The histological changes suggest that the tissues were prepared to withstand the increased external forces, in addition to create possibly additional skin in the relatively short term although at the expense of plausibly the cosmetic outcomes such as the amount of hair growth per surface area.
4. The histological results in control and expanded group exhibit changes in the dermis, epidermis and the distribution of the cells within two groups.
5. TrkB immuno- positive cells were comparable in control and expanded groups.
6. Nestin immune-positive markers were expressed in the rat expanded skin.

In conclusion, with the presence of nestin on the expanded skin at the end of 4 weeks, after maximum expansion state using anisotropic tissue expander, there is a tendency towards nerve present on the expanded skin. The findings of this study suggested that technique for expanding skin and soft tissue will generate additional skin tissue with similar quality, texture , color and innervation.

6.2 Recommendations

(1) Different methods for antigen retrieval procedures

Most formalin-fixed tissues require an antigen retrieval step before immunohistochemical staining. Methylene bridges formed during fixation cross-link proteins

and mask antigenic sites. Antigen retrieval methods break these methylene bridges and expose antigenic sites, allowing antibody to bind. Two methods for antigen retrieval are heat induced epitope retrieval (HIER) and enzymetic retrieval.

Enzymatic retrieval can sometimes damage the morphology of the section, so the concentration and treatment time need to be optimize. For enzymatic antigen retrieval, it is recommended to use the Trypsin solution, Pepsin solution or Proteinase K solution kit. Two methods for applying the solution to the tissue via; direct pippet or placing a rack of tissue slides into container of enzyme solution.the first method uses less reagent but each slide needs to be handled individually, the incubation time needs to be monitored carefully to ensure all slides are receiving the same treatment. It is easier to treat larger batches of slides by immersing them in a container of enzyme solution.

Heat-induced epitope retrieval is most often performed using a pressure cooker, a microwave, or a vegetable steamer. Some labs use a water bath set to 60 °C and incubate the slides in retrieval solution overnight. This is useful when working with the tissue sections that fall off the slide when heated at higher temperature; in particular bone, cartilage and skin.

(2) Different protein markers for future study

The recent study had point to the possibility of nerve exist on the expanded skin rat based on positive uptake of nestin markers on basal layer of epidermis and hair follicles within the dermis layer, however, there is a need to use other markers to clarify the presence of nerve on the expanded skin, either new nerve or pre-existing nerve.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Published works as well as papers presented at conferences, seminars, symposiums etc pertaining to the research topic of the research report/ dissertation/ thesis are suggested be included in this section. The first page of the article may also be appended as reference.