UNIVERSITI PUTRA MALAYSIA

CLINICAL, MICROSCOPIC AND MECHANICAL EVALUATION OF EXPANDED SKIN IN HORSES FOLLOWING SUBCUTANEOUS IMPLANTATION WITH ANISOTROPIC TISSUE EXPANDERS

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By

SADDAM HUSSEIN MOHAMMAED SALEH

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CLINICAL, MICROSCOPIC AND MECHANICAL EVALUATION OF EXPANDED SKIN IN HORSES FOLLOWING SUBCUTANEOUS IMPLANTATION WITH ANISOTROPIC TISSUE EXPANDERS

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February 2022

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Skin expander is a surgical an implant used to stretch cutaneous tissues through a combination of creep and biological stretch processes in other to generate extra cutaneous tissues for potential use in reconstructive skin surgeries. The understanding of the mechanical and histological features of expanded skin in horses is an important step to validate the use of tissue expander for skin extension in skin defect repair in horses. Therefore, this study aims to evaluate the clinical, mechanical, and histological properties of expanded skin following implantation of unidirectional anisotropic subcutaneous tissue expanders at different locations of horse's skin. In addition to that, possible behavioural changes in horses post tissue expander implantations were also investigated. A total of six horses were surgically implanted subcutaneously with unidirectional anisotropic tissue expanders at three different locations: the frontal region of the head, lateral side of the right shoulder, and dorsomedial part of the cannon region of the right forelimb. Each horse was clinically examined, and behaviour patterns were recorded in the stable box during the day and night-time, three days prior to - and six-days post implantation. After 14 days of subcutaneous expansion, skin biopsies of the expanded skin and representative normal skin samples from all the regions were collected. A part of each sample was subjected to mechanical properties study while the remaining was used for histological and immunohistochemical study. All horses tolerated the tissue expander implantation with no effect on the vital parameters as well as the overall horse behaviour. The mechanical properties of expanded skin from the shoulder area showed higher (11.57±1.23 MPa) elastic modulus (EM), maximum stress (MSs) (17.54±3.45 MPa), maximum strain (MSr) (177.70±58.53 %), and maximum force (MF) (150.70±59.89N), than the normal skin when compared to the forehead and lower forelimb. The overall effect of locations on EM and MSs was statistically significant ($p < 0.05$), however, there was no overall effect of horse factor, treatment factor (normal and expanded skin), and location interaction on the EM, MSS, MF, and MSr. The locations of the expanded or normal skin has effect on the EM and MSs with expanded skin from the frontal and distal limb areas been stiffer (less elastic) and need higher force to failure compared to the expanded skin of the shoulder. Histological evaluation revealed an increase in the thickness of the epidermal, dermal, and total skin layers with up regulation of vascular endothelial growth factor (VEGF) expression in the expanded skins. There was no significant difference in the histo-morphometric data between the three locations. The collagen fibres were more loosely packed in the expanded skin samples. The expression of VEGF was higher in the expanded skin than the normal skin. In conclusion, the implantation of unidirectional anisotropic subcutaneous tissue expanders in horses resulted in successful skin expansion with no physiological and behavioural discomfort. These findings will serve as important information when tissue expansion technique is to be applied in horses for skin reconstructive surgery according to anatomical regions. In general, skin expansion technique is considered a good technique to generate additional cutaneous tissue for equine skin reconstructive surgery.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENILAIAN KLINIKAL, MEKANIKAL DAN MICROSCOPIC KULIT YANG DIPERBAHARUI DALAM MODEL KUDA BERIKUTAN IMPLANTASI SUBKULITAN TISU PENGEMBANG ANISOTROPIK

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Pengembang kulit ialah implan pembedahan yang digunakan untuk meregangkan tisu kutaneus melalui gabungan proses regangan rayapan dan regangan biologi yang lain untuk menjana tisu kutaneus tambahan untuk kegunaan yang berpotensi dalam pembedahan kulit rekonstruktif. Pemahaman tentang ciri mekanikal dan histologi pengembangan kulit pada kuda merupakan langkah penting untuk mengesahkan penggunaan tisu pengembang untuk menyambungkan kulit dan membaiki kecacatan kulit pada kuda. Oleh itu, kajian ini bertujuan untuk menilai sifat klinikal, mekanikal dan histologi kulit yang mengembang berikutan implantasi pengembang tisu subkutaneus anisotropik satu arah di lokasi berbeza pada kulit kuda. Di samping itu, perubahan tingkah laku pasca implantasi tisu pengembang kuda juga telah disiasat. Sebanyak enam ekor kuda telah melalui pembedahan secara subkutan dengan implantasi tisu pengembang anisotropik satu arah di tiga lokasi berbeza: bahagian hadapan kepala, bahagian sisi bahu kanan, dan bahagian dorsomedial kawasan keting pada kaki depan kanan. Setiap kuda telah diperiksa secara klinikal, dan corak tingkah laku direkodkan dalam kandang masing-masing pada waktu siang dan malam, tiga hari sebelum - dan enam hari selepas implantasi. Selepas 14 hari pengembangan subkutaneus, biopsi kulit bagi kulit yang mengembang dan mewakili sampel kulit normal dari semua kawasan telah dikumpulkan. Sebahagian daripada setiap sampel telah tertakluk kepada kajian sifat mekanikal kulit manakala selebihnya digunakan untuk kajian histologi dan imunohistokimia. Semua kuda bertolak ansur dengan prosedur implantasi tisu pengembang tanpa memberi perubahan kepada parameter penting dalam tingkah laku kuda secara keseluruhan. Sifat mekanikal kulit mengembang dari kawasan bahu menunjukkan (11.57±1.23 MPa) modulus elastik (EM), tegasan maksimum (MSs) (17.54±3.45 MPa), ketegangan maksimum (MSr) (177.70±58.53 %), dan maksimum daya (MF) (150.70±59.89N) yang lebih tinggi, daripada kulit biasa jika dibandingkan dengan dahi dan bawah bawah. Kesan

keseluruhan lokasi pada EM dan MS adalah signifikan secara statistik ($p < 0.05$), walau bagaimanapun, tiada kesan keseluruhan pada faktor kuda, faktor rawatan (kulit normal dan mengembang), dan interaksi lokasi pada EM, MSS, MF, dan MSr. Lokasi kulit yang mengembang atau normal mempunyai kesan pada EM dan MS dengan kulit yang mengembang dari bahagian depan dan bahagian anggota distal adalah lebih kaku (kurang anjal) dan memerlukan daya yang lebih tinggi untuk gagal berbanding dengan kulit bahu yang mengembang. Penilaian histologi mendedahkan peningkatan dalam ketebalan lapisan epidermis, dermis, dan jumlah kulit dengan peningkatan regulasi ekspresi faktor pertumbuhan endothelial vaskular (VEGF) dalam kulit yang mengembang. Tidak terdapat perbezaan yang signifikan dalam data histo-morfometrik antara ketiga-tiga lokasi. Gentian kolagen tersusun lebih longgar dalam sampel kulit yang mengembang. Ekspresi VEGF lebih tinggi pada kulit yang mengembang daripada kulit biasa. Kesimpulannya, implantasi tisu pengembang subkutaneus anisotropik satu arah pada kuda Berjaya menghasilkan pengembangan kulit yang tanpa ketidakselesaan fisiologi dan tingkah laku. Penemuan ini akan menjadi maklumat penting apabila teknik pengembangan tisu akan digunakan pada kuda untuk pembedahan rekonstruktif kulit mengikut kawasan anatomi. Secara umumnya, teknik pengembangan kulit dianggap sebagai teknik yang baik untuk menghasilkan tisu kulit tambahan untuk pembedahan rekonstruktif kulit kuda.

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This is to confirm that:

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CBCT	Cone Computed Topography
CM	Centimetre
Diff	Difference
DM	Dorsomedial
DP	Dorsopalmar
DPX	Dibutylphthalate Polystyrene Xylene
EM	Elastic modulus
HBOT	Hyperbaric oxygen therapy
H&E	Haematoxylin and Eosin
IACUC	Institutional Animal Care and Use Committee
I.M	Intramuscular
ISO	International Organization for Standardization
IU	International Unit
I.V	Intravenous
Kg	Kilogram
LHL	left hind limb
LO	Lateral Oblique
MF	Maximum force
mm	Millimeter
mmHg	Millimeter high at 0°C
MMA	Methyl Methacrylate
MSs	Maximum stress

MSr	Maximum strain
OSMED	Hydrogel that uses the osmotic principle to gain volume
PBS	Phosphate Buffered Saline
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PMM	Polymethyl Methacrylate
RHL	Right hind limb
S.C	Subcutaneous
S.D	Standard Deviation
SPSS	Statistical Package for Social Science
STED	Soft tissue expansion device
UPM	Universiti Putra Malaysia
VEGF	Vascular endothelial growth factor
VEGF-R1	Vascular endothelial growth factor receptors 1
VEGF-R2	Vascular endothelial growth factor receptors 2
VP	Vinyl Pyrrolidone

CHAPTER 1

INTRODUCTION

1.1 Research Background

The skin is the largest organ of the horse body. It regulates temperature, comforts the horse with the sense of touch and protects against the immediate environment (Wong *et al.,* 2005, Lim and Nusse, 2013). Other functions of skin include excretion, resorption, and metabolism. The main defensive function of the skin is the homeostasis maintenance that controlled loss of water, ions and serum proteins (Elias and Wakefield, 2010, Darlenski *et al.,* 2011). However, the protective function of the skin from environmental stresses such as dehydration, irradiation, mechanical trauma, and pathogenic infection is through the aid of stratified epithelia lining it. Furthermore, the hair and sebaceous glands help in thermoregulation (Lim and Nusse, 2013).

Nevertheless, horses are prone to traumatic injury because of their fright and flight response and the nature of its environment that exposed horses to injury such as nail, metals or sharp object at the stables or surroundings. The resulting wound can be small or large. Managing small wound is not a problem because it undergoes normal healing process of inflammation, repair and maturation (Baxter, 2004, Knottenbelt, 2013, Theoret and Schumacher, 2017). In the case of large open wound mostly due to accident, burn or dead space following surgical removal of tumour growth, reconstructive surgery is usually required (Zöllner *et al.*, 2012a). Reconstructive surgery is an option to surgically replace the wound tissues with viable tissues. This is done for the purposes of anatomical restoration or cosmetic reasons.

Tissue expansion technique is a type of reconstructive surgery where tissue expander is used to mechanically generate excess soft tissue adjacent to a defect. The significance of this technique is that it allows replacement of loss tissue with analogous tissue with matching texture, colour and other important local features (Austad and Rose, 1982, Argenta *et al.,* 1983b, Radovan, 1984, Pisarski *et al.,* 1998, Agrawal and Agrawal, 2012, Tepole *et al.,* 2012). The process by which the expanded skin tissue keep the same structural and morphological features of the skin collagen and elastin fibers is term mechanical creep (Wilhelmi *et al.*, 1998). The application of tissue expanders was first carried out in a reconstructive surgery as early as 1900 but the clinical trial of the phenomenon was first described in humans, where an inflated rubber balloon was implanted subcutaneously to gain more skin tissue for the reconstruction of an external ear defect (Neumann, 1957). Decades later, the expansion process for breast reconstruction was pursued (Radovan, 1982). The application of tissue expanders in plastic reconstructive surgery has also been exploited by orthopaedic surgeons (Argenta *et al.*, 1983a, Argenta *et al.*, 1983b, Radovan, 1984). The first attempt to modify the conventional tissue expansion for better result was documented in 1984 (Sasaki and Pang, 1984). Since then, this technique became of immense value and added advantage over the flap surgery technique.

The fact that tissues have the ability to stretch made the application of tissue expanders an accepted practice over the last century in humans particularly in breast reconstruction, paediatric plastic surgery and other skin restorations (Cordeiro and McCarthy, 2006, Yeşilada *et al.*, 2013). Lately, the clinical application of skin tissue expansion was proven useful and it has been performed routinely for repair and reconstruction of skin defects in cosmetic surgery in human (McCauley, 2005, Motamed *et al.,* 2008, Fochtmann *et al.,* 2013, Lei *et al.,* 2015, Wang *et al.,* 2016).

The mechanism of creating additional skin by this protocol, and the physiologic changes occurring in the skin during the process of expansion have been studied in animal models (Sasaki and Pang, 1984). The implantation of silicone device tissue expanders subcutaneously in horses and dog to generate addition skin flap to repair defect have been described (Madison *et al.*, 1989).

However, despite the success of skin expansion with tissue expanders, its application in equine reconstructive surgery is yet to received attention. Most probably, the issues of the traditional type of the tissue expanders need to be addressed if this technique is to be applied clinically in the equine practice.

1.2 History of the use of tissue expanders

Recently, tissue expansion is considered one of the most significant innovative techniques in the reconstructive surgery field. The first reported tissue expansion utilization in reconstructive plastic surgery was in human in 1957 where an artificial subcutaneously implanted air-filled rubber balloon was inflated from outside the host body. In the year 1960 the use of hydrogels was suggested by Wichterle and Lim where it was used in a biomedical application (Wichterle and Lim, 1960). It was noted that hydrogel possessed the ability to swell, but it does not dissolve in aqueous media.

The use of a controlled slow expansion technique that require inflating silicon balloon with a saline solution through the skin filling port has been reported (Austad and Rose, 1982, Radovan, 1982, 1984). The high stretch response of silicon made it an important material for this technique. The mechanism of tissue expansion by controlled slow expansion technique is based on the osmotic gradient created by the saline (i.e. sodium and chloride) on the implanted silicon wall. Therefore, to provide an osmotic driving force the solute should be non-toxic and hypertonic (Austad and Rose, 1982). It has also been noted that the technique results in instant intraoperative tissue expansion where the skin stretches and widens in surface area (Sasaki, 1984).

Methyl Methacrylate (MMA) and Vinyl-pyrolidone (VP) were another type of osmotic driven tissue expander used for tissue expansion. They have been used extensively in the repair of cleft palate, breast reconstruction and congenital anophthalmia (Cordeiro and McCarthy, 2006, Gundlach *et al.,* 2005, Yeşilada *et al.,* 2013).The MMA and VP possess ability to absorb body fluids, thus increasing the size of the dry gel and causing expansion of the tissue without any practical external intervention (Wiese, 1993). However, the excessive rapid uncontrolled expansion which may lead to tissue necrosis became a limitation to their uses (Chummun *et al.*, 2010).

In order to minimize the possible complications associated with uncontrolled tissue expanders, co-polymer based hydrogel expander which allows considerable self-inflating expansion has been newly developed and its animal pre-clinical trials was tested in pigs (Swan *et al.*, 2012). The use of anisotropic self-inflating tissue expander that has the ability to expand unidirectional gave a positive outcome when tested in a craniofacial plastic reconstructive skin procedure (Swan *et al.*, 2011). Research on mechanical properties of expanded skin from animals such as pig, goat, sheep, and dog have been published (Zeng *et al.,* 2004, Zhang *et al.,* 2006, Manssor *et al.,* 2016). Although, in horses, there have been studies of mechanical behaviours of soft tissues particularly tendon and ligament (Souza *et al.*, 2010, Thorpe *et al.*, 2010), the investigation of skin expansion and its mechanical properties is relatively new in equine clinical research (Al-Majhali *et al.*, 2018, Whittaker *et al.*, 2020).

1.3 Problem Statement

In equine clinical setting, common surgical coverage of large wound at the distal part of horse's legs and face cannot be easily performed due to inadequate skin for closure (Zöllner *et al.*, 2012b). Therefore, large wound in horses has mostly been manage as an open wound, thus the delay in healing period with scar formation resulting in deformation of the injured area. The physiologic changes occurring in the skin during the process of expansion have been studied (Sasaki and Pang, 1984, Maher and Kuebelbeck, 2018). However, considering the high success rate of tissue expander in human plastic surgery, its use in veterinary medicine will ought to have recived some level of attention. Currently, research regarding the application of tissue expander to repair defect in animals is scarce (Madison *et al.*, 1989).

The issue of traditional expanders had been debated in clinical application in equine practice and the use of tissue expanders have been proposed as an alternative technique to skin grafting (Whittaker *et al.,* 2020). It is therefore expected that its application in equine clinical practice will offer an effective alternative to traditional wound grafts.

1.4 Justification of study

To date, an understanding of the mechanical and histological features of expanded skin is required to validate the use of tissue expander for skin extension in skin defect repair in horses. It is therefore our expectation that the investigation into mechanical, histological and immunohistochemical properties of expanded skin using tissue expander is the most common parts of horses susceptible to injury will enrich our understanding of the factors associated with using tissue expansion in managing large wound in horses. Furthermore, there is a need to determine any possible behavioural response to the tissue expander implantation. This is very important to understand if there will be tolerance to the procedure in the horse since the implant will beleft for sometime usually two or more weeks to allow for the expansion of the skin tissue. Afterall, behaviour response is one important factor used to measure animal welfare (Budras et al., 2012, Chung et al., 2018). Moreso, this will be the first study to assess behavioural changes in horses during tissue expansion process. Therefore, any deviation from the normal or usual horse behaviour following the implantation of tissue expander may render the procedure unfavourable for application in the horse. Hence, the results of this study will offer insights to the future developments and application of tissue expander in the field of cosmetic surgery in horse species.

Tissue expander has serve as an excellent strategy to grow skin without changing skin color, texture, hair balance, and thickness of the surrounding healthy skin. More importantly, the cells of expanded tissue have been shown to differentiate normally while keeping its characteristic phenotype on both histological and Immunohistochemical analysis. Furthermore, the use of tissue expander has reduces the incidence of infections associated of skin grafting and speed up the process of healing wounds without scar.

An anisotropic hydrogel tissue expander which is capable of controlled expansion against the uncontrolled expansion associated with an anisotropic expander will be used in this study. Its application in equine reconstructive surgery will help to ameliorate the challenges associated with closure of large wounds, which are most difficult to achieve.

1.5 Research hypotheses

Hypothesis 1

Ho: The mechanical properties of expanded skin due to subcutaneous implantation of anisotropic tissue expander is different from that of normal skin.

Ha: The mechanical properties of expanded skin due to subcutaneous implantation of anisotropic tissue expander is not different from that of normal skin.

Hypothesis 2

Ho: There are no behavioual changes to subcutaneous implantation of anisotropic tissue expander in horses.

Ha: There are behavioual changes to subcutaneous implantation of anisotropic tissue expander in horses.

Hypothesis 3

Ho: There are no difference in the histomorphometric features and expression of vascular endothelial growth factor (VEGF) between expanded and normal skin in the horses.

Ha: There are difference in the histomorphometric features and expression of vascular endothelial growth factor (VEGF) between expanded and normal skin in the horses.

1.6 Aim and objectives

1.6.1 Main aim

The main aim of this study is to investigate the clinical, mechanical, and histological properties of expanded skin at different locations in the horse.

1.6.2 Specific objectives

i. To investigate the possible behavioural changes in horses towards implantation of tissue expander subcutaneously.

ii. To determine the mechanical properties of expanded skin from different locations in horses following subcutaneous anisotropic self-inflating tissue expander implantation.

iii. To evaluate histo-morphological changes in expanded skin of horses in comparison to non-expanded skin.

1) To study the histological properties of the cutaneous tissue affected by implantation of self-inflating tissue expanders at different sites of horse skin.

2) To test the occurrence and assess the expression of the vascular endothelial growth factor (VEGF) in tissue expanded at different sites of horse skin.

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