



UNIVERSITI PUTRA MALAYSIA

***EFFECTS OF COLLAGEN-CALCIUM ALGINATE COMPOSITE
FILM WITH THERAPEUTIC ULTRASOUND MASSAGE ON OPEN
WOUND IN RATS AND CATS***

KHALED. M. A. HUSSIN

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**EVALUATION OF COLLAGEN-CALCIUM ALGINATE COMPOSITE
FILM WITH THERAPEUTIC ULTRASOUND MASSAGE ON OPEN
WOUND IN RATS AND CATS**

BY

Khaled M.A. Hussin

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

July 2014

ABSTRACT

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of doctor of philosophy

EVALUATION OF COLLAGEN-CALCIUM ALGINATE COMPOSITE FILM WITH THERAPEUTIC ULTRASOUND MESSAGE ON OPEN WOUND IN RATS AND CATS

By

KHALED. M. A. HUSSIN

July 2014

Chair: Associate Professor Dr. Jalila Abu, PhD

Faculty: Veterinary Medicine

This study was conducted with the hypothesis that collagen-calcium alginate film and therapeutic ultrasound massage are effective wound healing stimulants causing early re-epithelialization and uncomplicated wound healing. The objective of this study was to identify and evaluate wound healing properties of collagen-calcium alginate film and therapeutic ultrasound massage on open wounds in rats. One hundred and twenty healthy Sprague-Dawley rats were used in the study. Ketamine (5 mg/kg) and Xylazine (50 mg/kg) combination was used. A skin wound (2x2cm) was created lateral to the spine between the base of the scapulae and the iliac crest. The rats were randomly divided into four groups (n = 30) namely Group I (control group), Group II (treated with therapeutic ultrasound massage), Group III (treated with collagen-calcium alginate film) and Group IV (treated with collagen-calcium

alginate film with therapeutic ultrasound massage). Evaluation on the effect of biomaterials on open wound was based on clinical observation, haematological, bacteriological, biochemical and histopathological examinations on day 4, 8, 12, 16 and 20 (post-wounding).

The collagen-calcium alginate films with therapeutic ultrasound massage were well accepted and tolerated by animals and did not cause any adverse reactions. Animals in Group IV showed bright red granulation tissue, without malodour and exudates on day 20 post-wounding. This group also had a higher mean percentage of wound epithelialization, wound contraction, and total wound healing was significantly higher ($P < 0.05$) compared to the other group. *Staphylococcus aureus*, *Klebsiella* spp., *Proteus* spp. and *Pseudomonas* spp. were isolated from all animals. Wound of surgical or traumatic origin is invariably colonized by bacteria. Among the common micro-organisms associated with wound infection are *Staphylococcus aureus* and beta-haemolytic *Streptococcus* which are considered as transient flora of the skin. Day 8 post-wounding, typical histological appearance of mature fibroblasts with relatively loose type of supporting tissue was noticed. The fibroblast nuclei were elongated in the direction of the collagen fibers with neovascularization in animals in group IV. The predominant nucleoli reflect active protein synthesis in wound healing.

Clinically Collagen-calcium alginate film with therapeutic ultrasound massage was effective when used in rats, it is used in clinical cases involving 8 cats presented at Progressive Animal Welfare society (PAWS).. All the cats. No animals showed any

intolerance and disturb did not the bandage throughout the treatment. Formation of healthy granulation tissue was observed in all the cases without any side effects.

As a conclusion, collagen-calcium alginate film with therapeutic ultrasound massage was an effective wound healing stimulant. This biomaterial combination also was found to be better than Collagen-calcium alginate film or therapeutic ultrasound massage alone. The findings have a commercial application because it is inexpensive alternative to stimulate wound healing in animals where the cost of treatment is a major concern.

ABSTRAK

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk ijazah Doktor Falsophy

PENILAIAN KOLAGEN KALSIMUM ALGINAT FILEM KOMPOSIT DENGAN URUTAN ULTRASOUND TERAPEUTIK PADA PENYEMBUHAN LUKA TERBUKA PADA TIKUS DAN KUCING

Oleh

KHALED. M. A. HUSSIN

July 2014

Pengerusi: Profesor Madya Dr. Jalila Abu, PhD

Fakulti: Perubatan Veterinar

Kajian ini dijalankan dengan hipotesis yang filem kolagen-kalsium alginat dan urutan terapeutik ultrabunyi adalah perangsang penyembuhan yang berkesan disebabkan pertumbuhan kulit seperti pada asalnya dan pemulihan luka yang efektif. Objektif kajian ini adalah untuk mengenal pasti dan menilai keberkesanan filem kolagen-kalsium alginat dan urutan terapeutik ultrabunyi dalam merawat luka pada tikus.

Sebanyak 120 tikus jenis 'Sprague-Dawley' telah digunakan dalam kajian ini. Campuran Ketamine (5 mg/kg) dan Xylazine (50 mg/kg) telah digunakan. Satu kawasan luka kulit (2x2 cm) telah buat disisi tulang belakang antara pangkal 'scaplae' dan 'iliac crest'. Tikus dibahagi secara rawak kepada empat kumpulan. Kumpulan I (kumpulan yang dikawal), Kumpulan II (dirawat menggunakan urutan terapeutik ultrabunyi), Kumpulan III (dirawat menggunakan filem kolagen – kalsium alginat) dan Kumpulan IV (dirawat menggunakan urutan terapeutik ultrabunyi serta

menggunakan filem kolagen – kalsium alginate). Ujian keberkesaan biobahan pada luka telah dijalankan secara pemerhatian klinikal, hematologi, bakteriologi, biokimia dan histopathologi pada hari 4, 8, 12, 16 dan 20 (selepas luka).

Kolagen-kalsium alginat dengan urutan terapeutik ultrabunyi diterima dengan baik dan sesuai dan tidak menyebabkan reaksi pada kulit. Kumpulan IV mempamerkan kemerahan tisu granulat tanpa bau busuk dan eksudat pada hari 20 selepas luka. Kumpulan ini juga menunjukkan peratus tertinggi dalam epiteliasasi luka, pengecutan luka dan pemulihan keseluruhan yang signifikan ($P < 0.05$) berbanding kumpulan yang lain.

S. aureus, *Klebsiella* spp, *Proteus* spp. dan *Pseudomonas* spp. telah dijumpai dalam haiwan di setiap kumpulan. Kadar kecekapan formasi ‘fibroblast’ dan ‘angiogenesis’ di dapati berkesan dalam Kumpulan IV. Ia merangsang sembuh awal (hari 8). Ujian klinikal mendapati filem Kolagen-kalsium dengan urutan terapeutik ultrabunyi didapati amat berkesan dalam lapan ekor kucing. Semua kes-kes. Haiwan menunjukkan sebarang sikap tidak bertoleransi dan tidak mengganggu balutan sepanjang tempoh rawatan. Pembentukan tisu granulation sihat diperhatikan dalam semua kes tanpa sebarang kesan sampingan.

Sebagai rumusan, Kolagen-kalsium dengan urutan terapeutik ultrabunyi didapati adalah perangsang penyembuh luka yang berkesan. Campuran biobahan ini juga dapati lebih berkesan berbanding dengan filem Kolagen-kalsium alginat dan urutan terapeutik ultrabunyi yang di jalankan secara berasingan. Hasil kajian ini, mempunyai aplikasi komersial kerana ia adalah alternatif yang murah bagi

merangsang pemulihan luka haiwan di mana harga menjadi aspek penting pada pelanggan.



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APPROVAL SHEETS

I certify that a Thesis Examination Committee has met on **08 July 2014** to conduct the final examination of **KHALED. M. A. HUSSIN** on his thesis entitled "**EVALUATION OF COLLAGEN-CALCIUM ALGINATE COMPOSITE FILM WITH THERAPEUTIC ULTRASOUND MASSAGE ON OPEN WOUND IN RATS AND CATS**" in accordance with Unversiti Pertanian Malaysia Colleges Act 1971 and the Constitution of the Unversiti Pertanian Malaysia [P.U. (A) 106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Rasedee @ Mat Bin Abdullah, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Malaika Watanabe, PhD

Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Shanthi Ganabadi, PhD

Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Sunderarajan Thilagar, PhD

Professor
Institute of Veterinary Education and Research
Rajiv Gandhi Institute
(External Examiner)

NORITAH OMAR, PHD

Assoc. Professor/ Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia
Date: 08 July 2014

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the supervisory committee were as follows:

Jalila Abu, PhD

Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Kalthum Hashim, PhD

Professor
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Member)

Noordin Mohamed Mustapha, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PHD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia
Date: 08 July 2014

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Signature: _____
Name of
Chairman of Supervisory
Committee: Associate Professor Dr.
Jalila Abu, PhD

Signature: _____
Name of
Member of Supervisory
Committee: Professor Dr.
Kalthum Hashim, PhD

Signature: _____
Name of
Member of Supervisory
Committee: Professor Dr.
Noordin Mohamed, PhD

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LIST OF ABBREVIATIONS

-	Negative
%	Percentage
+	Positive
AD	Anno Domini
ANOVA	Analysis of variance
BC	Before Christ
BCA	Bicinchonic acid
BSA	Bovine Serum Albumin
°C	Degree Celsius
DPX	A mixture of Distyrene, Plasticier & Xylene
<i>E. coli</i>	<i>Escherichia coli</i>
E.C.M	Extracellular matrix
EDTA	Ethylene Diamine Tetra-Acetate
G	G-force
H&E	Haematoxylin & Eosin Haemoglobin
Hb	Haemoglobin
IM	Intramuscular
kDa	Kilodalton
Kg	Kilogram
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
µg	Microgram
MHz	Megahertz

μm	Micrometer
μl	Microlitre
MT	Masson Trichrome
OD	Optical Density
PAWS	Progressive Animal Welfare Society
PCV	Packed Cell Volume
pH	Reciprocal of the hydrogen ion concentration
RBC	Red Blood Cell
SD	Standard Deviation
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
TUM	Therapeutic Ultrasound Massage
US 5X	Received Ultrasound Massage applications soon after wounding, and 4, 8, 12 and 16 days later
WBC	White Blood Cell

CHAPTER 1

INTRODUCTION

The largest organ of the body is skin since nearly 15% of the overall body weight is of body's skin. It refers to the complex organ which covers the entire body surface and exists in continuity with the lining of the body openings i.e. mucous membranes (Cribier and Grosshans, 2002; McKee, 1999). Skin has several important roles in the body as it is involved in sensory functions, thermoregulation, and synthesis of vitamin D and storage of nutrients like proteins, carbohydrates, fats and water. It also demonstrates a protective role by acting as a barrier to radiations, desiccation and chemicals (Anderson, 1997; Thilagar *et al.*, 2009). Skin is considered to be the most affected part in case of traumatic injuries (Prathiba and Gupta, 2000). Any disruption to standard continuity of the structure of body is termed as a wound. It can be caused by thermal, mechanical or physical agents. In small animal practice, traumatic wounds are commonly encountered and delayed healing of these wounds is not only costly but also disturbing for the affected individuals (Anderson, 1996; Dart *et al.*, 2002b).

Wound healing serves to be a highly complicated but sophisticated organized biological phenomenon. It involves a series of biochemical, molecular, physiological and cellular interactions which are essentially required by the restoration of the anatomical integrity of the injured part. In this way it stops pathogenic microbes to invade the tissues (Raghow, 1994; Soni, 2013) and helps in restoring the typical structural continuity and efficiency (Prathiba and Gupta, 2000).

The chief targets of wound management include functional restoration, pain relief, cosmetic repair, employment of efficient techniques and sensible timely decisions if there is any delay in wound healing (Anderson, 1996; Cockbill, 2002). For managing wounds of humans as well as animals, products like growth factors with cellular activity are under development which would be utilized for strengthening single or multiple processes of the wound healing pathway (Cockbill, 2002). Considering the cost-effectiveness, recipient's satisfaction and convenient use of these products, they would prove to be efficient for novel dressings as well.

The process of wound healing can be divided into three phases.

I: Inflammatory and debridement phase which lasts for 2-5 days after injury. During this phase, monocytes, fibroblasts and neutrophils migrate to the damaged tissue.

II: Proliferative or repair phase which lasts for 2-3 ensuing weeks. During this phase, wound macrophages get activated leading to synthesis of extracellular matrix and collagen, proliferation of fibroblasts and endogenous growth factor production.

III: Maturation phase which duration may take 2 weeks to one year after injury. It involves wound remodeling leading to production and cross-linking of active collagen (DeRossi *et al.*, 2009; Hosgood, 1993). The stages of wound healing are shown in Figure 1.1.

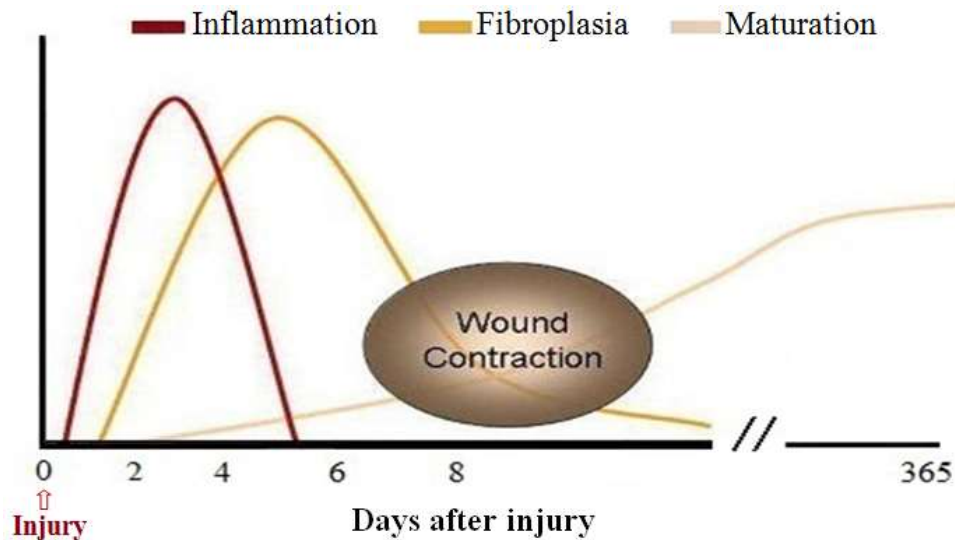


Figure 1.1. Stages of wound healing
(Georgie, 2007)

The usual sequence of the wound healing process is disturbed in case of chronic wounds because of imperfect remodeling of extracellular matrix, continuity of inflammation for long time and inability to re-epithelialise (James and Bayat, 2003). A vital element of wound management is prompt coverage of wound (Singer and Clark, 1999). In addition to this, it is important to change the bandage regularly, especially in wound management in animals. A challenging task in this connection is to keep the bandage safe from contaminants such as urine (Anderson, 2003). Topical antiseptics are toxic to fibroblast and epithelial cells. Topical antibiotics in infected wounds are likely to be washed off by exudates and therefore it is more effective to give systemic. Cleaning solutions or cream made of malic, benzoic and salicylic acids in propylene glycol have a very low pH of 2.4 and can be an irritant for the surrounding normal (Anderson, 2003). Dry gauze dressing used traditionally has been almost replaced by wound dressings such as hydropolymers, aligates, foams, gels and hydrocolloids. Speedy re-granulation of wounds is important to avoid

additional morbidity or mortality. Certain wounds don't respond to the typical wound healing methods and remain static (Ballard and Baxter, 2000).

Development of engineered skin and tissue has attracted significant level of interest and investment in wound management. In case of wounds of large size, a manufactured skin tissue can be used for wound coverage. However, a single graft can be as costly as £ 250- £1000 (Ballard and Baxter, 2000). Similarly, the cost of keratinocyte layers lie in the range of \$1,000 to \$13,000 (Ballard and Baxter, 2000). Besides being very costly, these engineered tissues need extraordinary conditions for storage and careful handling (Anderson, 2003).

Biological materials like natural or synthetic polymers or their composites are also employed for covering wounds temporarily if there is extensive skin damage. (Vedakumari *et al.*, 2013) Biomaterials which are employed for replacing a fraction of an organism or for developing a compatible contact with living tissue can either be natural or synthetic. Examples of natural biomaterials include those derived from omental pedicles (Brockman *et al.*, 1996; Smith *et al.*, 1995; Wang *et al.*, 2012), submucosa, small intestine and porcine (Holt and Mann, 2002). Whereas, examples of synthetic biomaterials include carbon-graphite fibres, bioglass and ceramics (Roush, 2003). Natural biomaterials like calcium alginate and collagen are vital tools in tissue regeneration which is utilized as stimulators of wound healing (Drury and Mooney, 2003).

Collagen is a class of proteins naturally present in animals, particularly in the connective tissues and flesh of vertebrates (Müller, 2003). Collagen is the chief

constituent of connective tissues and since it accounts for 25-35% of the total protein content of the mammal's body, it is considered to be the most abundant protein in them (Di Lullo *et al.*, 2002). The most common collagen producing cell is the fibroblast and is present in abundance in bone, cartilage, intervertebral disc, gut, cornea and blood vessels; however, it exists mainly in fibrous tissues like skin, ligament and tendon.

Collagen is the chief constituent of endomysium in muscles. It constitutes 6% of the weight of tendinous muscles of high strength. The collagen content of muscle tissues ranges from 1-2% (Sikorski, 2001). Hydrolyzed collagen and collagen gels are utilized in cosmetic applications and skin therapies as absorbents (Sharma, 2011). A good source of alginate is seaweed which is used to manufacture calcium alginate dressings (Knill *et al.*, 2004). In case of heavy exudates, these can be used instead of hydrocolloid dressings as calcium alginate dressings are highly absorbent (Jude *et al.*, 2007). Their absorptive capacity makes them ideal tool for not only surface dressing but also for filling cavities after cleaning or draining. The mechanism of action is the same as for hydrocolloid dressings i.e. they develop a natural gel comprising of exudates against the healing wound maintaining flexibility and humidity thus facilitating the tissue growth and healing process. Besides this, the gel also behaves as a barrier to microbial invasion which can lead to secondary infections and obscure wound healing (MSEC, 2013).

The procedure of changing dressings is the same as the one which is usually used involving rinsing and washing of wound (MSEC, 2013).

Considering the convenience in removal of calcium alginate dressings, they can also be used in burn cases. However, they are also employed in ulcer patients, especially for diabetic skin ulcers whose management has proved to be quite challenging with other dressings. While cleaning the wound, the skin is rinsed with saline or simply sloughs off with gel. In contrast to other dressings, it is important to keep the calcium alginate dressings away from normal tissue and apply to damaged tissue only for efficient results. Medical tape and gauze can be used to keep them in a suitable position (MSEC, 2013).

It has been established that therapeutic ultrasound (TUS) application speeds up tissue repair, relieves pain, causes muscle relaxation, enhances local blood flow, modifies scar development and decreases edema (Coakley, 1978; Da Costa Gonçalves *et al.*, 2007; Dyson, 1990; Dyson *et al.*, 1968; Maxwell, 1992; Monte-Raso *et al.*, 2005; Ter Haar, 1978; Young and Dyson, 1990).

Since ultrasound irradiation has been proven to increase skin regeneration (Dyson *et al.*, 1968; Levine and Watson, 2013; Young and Dyson, 1990), it is recommended for treatment of pressure ulcers (Da Costa Gonçalves *et al.*, 2007; Dyson and Suckling, 1978; Roche and West, 1984) and varicose ulcers (Da Costa Gonçalves *et al.*, 2007; Riet *et al.*, 1996). Utilization of ultrasound irradiation for improving the quality and speed of integration of skin grafts has been studied (Da Costa Gonçalves *et al.*, 2007; Ivanov, 1987); however, these activities require further investigation.

Topical products contain wound healing stimulators which cause activation of wound healing cells like macrophages for generating cytokines for early epithelialisation during the course of healing of wound (Swaim, 2003). Wound therapies are the main

focus of researchers exploring advanced wound management (Wendelken *et al.*, 2003). In this regard, utilization of biomaterials as a permanent substitute for skin is still a challenging task (Lin *et al.*, 2000).

The objectives of this research are as follows.

1. to evaluate the clinical effects of collagen-calcium alginate film and therapeutic ultrasound massage for open wounds in experimental animals.
2. to determine the presence of infection and changes in haematological values of animal with open wound treated with collagen-calcium alginate film and therapeutic ultrasound massage in experimental animals.
3. to evaluate the histopathological changes in open wound with collagen-calcium alginate film and therapeutic ultrasound massage in experimental animals.
4. to evaluate the effectiveness of the biomaterials in clinical cases presented at Progressive Animal Welfare society (PAWS).

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BIODATA OF STUDENT

Khaled was born in Benghazi, Libya on April 5, 1977. He is the 1st child of Mr. Masood A. Hussin. Khaled is happily married to Ms. Asma Saleh and they are blessed with two sons and one daughter, Tiam, Yazan and Aytin. He pursued his higher secondary school from, Asr El- gamaher school higher secondary school, Benghazi. After school education, in 1996 pursued his Bachelor of Veterinary Science from Omar Al- Mukhtar University, El Beida, Libya. After completion of B.V.Sc., he started work with the private sector in different veterinary fields for 18 months. In June 2004, he continued studying for a Master of veterinary medicine in University Putra Malaysia (UPM). Soon after completion of his Master study, he was transferred to the Department of Veterinary Surgery and Radiology as a Lecturer at Omar Al- Mukhtar University, El Beida, Libya in 2006 and served for two years. To pursue his Doctor of Philosophy degree (PhD) programmed in Veterinary Surgery, with scholarship. He joined the faculty of, Veterinary Medicine, University Putra Malaysia (UPM) in the year 2008. So far, he has published two citation journal papers during his PhD candidature and two others currently under review. He also published two papers from his MVM work and several publications, conferences and seminar thereafter.

LIST OF PUBLICATIONS

1. Khaled, M. A., Jalila, A., Kalthum, H., Noordin, M., and Asma, S. W. 2014. Effects of biomaterials collagen-calcium alginate film with therapeutic ultrasound for management on wound healing in a rat. Accepted for publication at *Journal of Animal and Veterinary Advances*.
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