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 MASSAGE 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 KALSIUM 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
cawasan 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

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

Kolagen-kalsium alginate 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 menunjukan peratus tertinggi dalam epiteliasasi luka, pengecutan luka dan pemulihan keseluruhan yang signifikan (P< 0.05) berbanding kumpulan yang lain.


Sebagai rumusan, Kolagen-kalsium dengan urutan terapeutik ultrabunyi didapati adalah perangsang penyembuh luka yang berkesan. Campuran biobahan ini juga dapat 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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In the Name of Allah, Most Gracious, Most Merciful, all praise and thanks are due to Allah, and peace and blessings be upon His Messenger. I would like to express the most sincere appreciation to those who made this work possible: Advisory members, Friends and Family.

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I wish to thank Dr. Gowry, Dr Kavitha, the manager and the entire staff of Progressive Animal Welfare Society (PAWS) for providing us with the research cats and technical support during sampling.

My thanks also go to my friend Dr Salisu Buhari for the moral and intellectual support and Dr Essam Rzawan, Mohammed Warg and Hatem Shakhtor for assistance in handling expt animal and blood sampling.

Thanks and acknowledgements are meaningless if not extended to my father's spirit and pure, mum and grandmother who deserve my deepest appreciation. I am grateful for the countless sacrifices they made to ensure that I could pursue my dreams and for always being there for me. Real and deepest thanks to them (May ALLAH bless
and protect them and may they be blessed with long and healthy life). All praise and thanks words said to them will not be enough.

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And last but not the least, I wish to thank my University (Omar Al- Mukhtar University, El Beida, Libya) and my country Libya for their contributions in my study either directly or indirectly.
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 Universiti Pertanian Malaysia Colleges Act 1971 and the Constitution of the Universiti Pertanian Malaysia [P.U. (A) 106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy.

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---

**BUJANG BIN KIM HUAT, PHD**  
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Date: 08 July 2014
DECLARATION

Declaration by graduate student

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- the research conducted and the writing of this thesis was under our supervision;

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Signature: ____________________________
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<tbody>
<tr>
<td>-</td>
<td>Negative</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>AD</td>
<td>Anno Domini</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BC</td>
<td>Before Christ</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchonic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>DPX</td>
<td>A mixture of Distyrene, Plasticer &amp; Xylene</td>
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<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<td>E.C.M</td>
<td>Extracellular matrix</td>
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<td>EDTA</td>
<td>Ethylene Diamine Tetra-Acetate</td>
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<tr>
<td>G</td>
<td>G-force</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin &amp; Eosin Haemoglobin</td>
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<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
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<tr>
<td>MCHC</td>
<td>Mean Corpuscular Haemoglobin Concentration</td>
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<td>MCV</td>
<td>Mean Corpuscular Volume</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<td>MHz</td>
<td>Megahertz</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>µm</td>
<td>Micrometer</td>
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<td>µl</td>
<td>Microlitre</td>
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<td>MT</td>
<td>Masson Trichrome</td>
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<td>OD</td>
<td>Optical Density</td>
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<td>PAWS</td>
<td>Progressive Animal Welfare Society</td>
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<td>PCV</td>
<td>Packed Cell Volume</td>
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<td>pH</td>
<td>Reciprocal of the hydrogen ion concentration</td>
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<td>RBC</td>
<td>Red Blood Cell</td>
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<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
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<td>TUM</td>
<td>Therapeutic Ultrasound Massage</td>
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<td>US 5X</td>
<td>Received Ultrasound Massage applications soon after wounding, and 4, 8, 12 and 16 days later</td>
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<td>WBC</td>
<td>White Blood Cell</td>
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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.
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).
REFERENCES


DeLapp, N. W., and Dieckman, D. K. 1990. Effect of basic fibroblast growth factor (bfgf) and insulin-like growth factors type i (igf-i) and type ii (igf-ii) on adult human keratinocyte growth and fibronectin secretion. *Journal of Investigative Dermatology*, 94(6): 777-780.


Kaufman, T., Levin, M., and Hurwitz, D. J. 1984. The effect of topical hyperalimentation on wound healing rate and granulation tissue formation of


APPENDICES

Appendix A approved of the proposed animal utilization protocol
Appendix B1

Blood agar base

- Beef heart infusion: 375g
- Tryptose: 10g
- Sodium chloride: 5g
- Agar: 15g
- Distilled water: 1000ml

Autoclaving was done at 121°C for 15 minute.

Cool agar (50°C) and add 53ml defibrinated bovine blood and pour into sterile Petri plate, 20ml/plate under sterile condition.

Appendix B2

MacConkey agar

- Peptone: 17g
- Proteose-peptone: 3g
- Lactose: 10g
- Bile Salts: 1.5g
- Agar: 13.5g
- Neutral red: 0.03g
- Crystal violet: 0.001g
- Distilled water: 1000ml

Autoclaving was done at 121°C for 15 minute.

Cool agar (50°C) and pour into sterile Petri plate, 20ml/plate under sterile condition.
Appendix B3

Gram stain

1. **Stock crystal violet**

   Crystal violet  
   Ethyl alcohol

2. **Stock oxalate solution**

   Ammonium oxalate  
   Distilled water

   Crystal violet working solution: Mix 20 ml of solution 1 with 80 ml of stock.

3. **Gram’s Iodine Solution**

   Iodine crystal  
   Potassium iodide

   Dissolve the above reagents in 10 ml of distilled water then add distilled water up to 200.0ml and store in dark bottle.

4. **Decolourizer**

   Acetone  
   Ethyl alcohol


5. Counter Stain

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tr>
<td>Basic Fuchsin</td>
<td>3.0g</td>
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<tr>
<td>Ethyl alcohol</td>
<td>100.0ml</td>
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</table>

**Procedure:**

STEP 1: Flood the entire slide with crystal violet and stand for about 60 seconds. Wash the slide for 5 seconds with water.

STEP 2: Flood the slide with the iodine solution for about a minute. Rinse the slide with water for 5 seconds.

STEP 3: Add the ethanol drop by drop until the blue-violet color is no longer emitted. Rinse the slide with water for 5 seconds.

STEP 4: Flood the slide with the safranin stand for about a minute to allow the bacteria to incorporate the saffranin (Gram negative bacteria, take on a pink color). Rinse the slide with water for 5 seconds.

STEP 5: Blot the slide gently with tissue paper or allow it air dry before viewing it under the microscope.
Appendix C1

Haematoxylin and Eosin staining

Harris haematoxylin

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tr>
<td>Haematoxylin</td>
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<tr>
<td>Absolute alcohol</td>
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</tr>
<tr>
<td>Ammonium or potassium sulphate</td>
<td>20 grams</td>
</tr>
<tr>
<td>Mercuric oxide</td>
<td>0.5 gram</td>
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<tr>
<td>Distilled water</td>
<td>200 ml</td>
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</table>

The haematoxylin was dissolved in alcohol and the ammonium in water with aid of heat; both the haematoxylin and the alum solution were combined and boiled. The addition of mercuric oxide made the solution dark purple in colour. The solution was filtered before being used or stored.

Eosin counters stain for haematoxylin 1% stock alcohol eosin

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tr>
<td>Eosin Y (water soluble)</td>
<td>1 Gram</td>
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<tr>
<td>Distilled water</td>
<td>20.0 ml</td>
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<tr>
<td>95% alcohol</td>
<td>80.0 ml</td>
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Working eosin solution

<table>
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<tr>
<td>Eosin stock solution</td>
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</tr>
<tr>
<td>80% alcohol</td>
<td>20.0 ml</td>
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</table>

Just before use, 0.5 ml of glacial acid was added to each 100 ml of stain and stirred.
Staining technique

1. De-wax section in xylene for 5 minutes.
2. Rehydrate in 100% alcohol and 70% each for 8 minutes.
3. Wash in running water by dipping up and down for 1-2 minutes.
4. Stain in Haematoxylin for 1-2 minutes.
5. Wash in running water until no trace of stain observed on the tissues.
6. Decolorize in 1% acetic alcohol by dipping 3 times.
7. Wash in running water for 10 minutes for bluing.
8. Stain in eosin for 30 seconds.
9. Dehydrate with 95% alcohol.
10. Clean in xylene.
11. Mount with coverslip using DPX.

Appendix C2
Masson’s trichrome stain

1. Bouin’s solution
   Picric acid saturated aqueous solution 750.00 ml
   Formalin 37-40% 250.00 ml
   Glacial acetic acid 50.00 ml

2. Weigerts’ iron haematoxylin solution
   Solution A:
   Haematoxylin crystals 1.00 grams
   Alcohol 95% 100.00 ml
Solution B:

Ferric chloride 29 % aqueous 4.00 ml
Distilled water 95.00ml
Concentrated Hydrochloric acid 1.00ml

Working solution

Equal parts of solution A and solution B

3. Biebrich Scarlet- Acid Fuchsin Solution

Biebrich Scarlet, aqueous 1% 90.00 ml
Acid Fuchsin aqueous 1 % 10.00 ml
Glacial acetic acid 1.00 ml

4. Phosphomolybdic-phosphotungstic Acid

Phosphomolybdic acid 5 grams
Phosphotungstic acid 5 grams
Distilled water 200 ml

5. Aniline blue solution

Aniline blue 2.5 grams
Glacial acetic acid 2.00 ml
Distilled water 100.00 ml

6. 1 % Acetic asid

Glacial acetic acid 1.00 ml
Distilled water 100.00 ml
Masson’s trichrome (MT) Staining technique

1. De-wax section in xylene for 5 minutes.
2. Rehydrate in 100% alcohol and 70% each for 8 minutes.
3. Wash in running water by dipping up and down for 1-2 minutes.
4. Slides submerged in Bouin’s fluid for 1 hour at 56˚C.
5. Wash in running water until no traces of stain observed on the tissues.
6. Stain in Weigert’s haematoxylin for 5 minutes.
7. Rinsed thoroughly in running tap water until no traces of stain observed on the tissues.
8. Stain in Biebrich scarlet-acid fuchsin for 15 minutes.
9. Rinsed thoroughly in distilled water.
10. Submerged in Phosphomolybdic/Phosphotungstic acid 10 to 15 minutes.
11. Submerged in Aniline blue stain for 5 to 10 minutes.
12. Rinsed thoroughly in distilled water.
13. The slides were placed in 1 % Acetic acid for 3 to 5 minutes.
14. Dehydrate with 95 % alcohol.
15. Clean in xylene.
16. Mount with cover slip using DPX.
Appendix D
The results of wound planimetry

<table>
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<tr>
<th>Case No</th>
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<th>2&lt;sup&gt;nd&lt;/sup&gt; (day 6)</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; (day 9)</th>
<th>4&lt;sup&gt;th&lt;/sup&gt; (day 12)</th>
<th>5&lt;sup&gt;th&lt;/sup&gt; (day 15)</th>
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NR- not reported.
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


