Evaluation of early wound healing events in a rat wound model treated with “active” topical dressings

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Abstract

Introduction: Chronic wounds are commonly associated with biofilms and exaggerated inflammation resulting in non-healing. "Bioflammacides" are a recently described group of compounds used in the treatment of chronic and recalcitrant wounds. "Bioflammacides" target biofilms and inflammation aiding a change in the wound environment enabling cutaneous wound healing.

Aim: This study aims to evaluate the effects of two targeted "active" topical dressings, ; a bioflammacide gel (Flavonix®), and a nanocrystalline silver sheet (Acticoat®), on the wound healing events in an *in vivo* rat chronic wound model.

Methods: A chronic wound model was created in 128 Sprague-Dawley rats, modifying previously described methods by combining burn and excisional wounds. Wounds were inoculated with a bacterial broth (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) on POD 4. The wounds were then assigned to the following treatment groups on POD 7; Flavonix®, Acticoat® and a negative control. An additional non-inoculated control group (no bacterial or other broth) was included. Eight animals were assigned to each group at each time point. The study was conducted over 21 days and the categorical variables assessed were epithelial gap, cellular proliferation at the wound edge at Days 10,14 and 21 and semi-quantitative culture for bacterial load at Days 10 and 21.

Results: Both Acticoat® and Flavonix® showed improved wound healing compared to the control group. Epithelial gap distances were significantly different between the Acticoat® group and the negative control group at Day 21 ($p = 0.0350$) (8mm vs. 12.8mm). Cellular proliferation profiles were most modulated in the Flavonix® treated group at Day 21 in

comparison to the negative control group (p=0.013)(1.45 vs. 8.65) Bacterial load based on semi-quantitative culture showed significant differences in *Pseudomonas aeruginosa* counts at POD 21 with all treatment groups except Acticoat® but failed to show a significant change with the *Staphylococcus aureus* counts in any groups.

Conclusion: Flavonix® and Acticoat® both demonstrated similar effects on wound healing events in our chronic wound model with significant differences being noted between the treated groups and negative controls in epithelial gap reduction and cellular proliferation profiles. Bacterial burden in the form of a mixed species biofilm was not convincingly altered by any of the treatment groups, but this did not alter the wound in its ability to close, suggesting that inflammatory balance plays an important role as a common pathway in cutaneous healing.

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List of Abbreviations

EPS – extra polymeric substance

DNA – double stranded nucleic acid

IL1 – interleukin 1

TNF-α - tumour necrosis factor alpha

IL6 – interleukin 6

ECM – extracellular matrix

MMP – matrix metalloproteinase

IL-1α - interleukin 1 alpha

POD – post operative day

V/V – volume/volume

po – per os

sc – subcutaneous

ip – intraperitoneally

rpm – revolutions per minute

NC – negative control

NIC – non-inoculated control

H&E – Haemotoxylin and Eosin

HIER – Heat Induced Epitope Retrieval

HPF – high power field

CFU's – colony forming units

BF - Bioflammacides

NCS – Nanocrystalline silver

PCR – polymerase chain reactions

Declaration

I, Chaitanya Chandrakant Patel declare that this research report is my own work. It is being submitted for the degree of Master of Science in Medicine in the branch of Plastic and Reconstructive Surgery, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signed at _____ on this _____ day of _____ 2013.

_____
Signature

To Terri, for all your patience, support and love in seeing this to its completion.

Presentations

2010

Annual Congress of the Association of Plastic and Reconstructive Surgeons of South Africa (APRSSA).
Title: Evaluation of early wound healing events when using topical “active” dressing in a rat wound model.

2011

The South African Association for Laboratory Animal Science (SAALAS).
Title: Development of a chronic wound model – lessons learnt

Introduction

Chronic cutaneous wounds, exemplified by diabetic ulcers, chronic venous ulcers and pressure sores pose a vastly significant socio-economic, health and personal problem the world over.[1] Diseases of modern living and excess have resulted in a higher incidence of these wounds, particularly due to the obesity epidemic, a sharp rise in diabetes and an ever-ageing population. There are over 6.5 million Americans afflicted with these types of wounds, with an annual treatment cost of 25 billion dollars. Average costs per patient for venous ulcers were found to be $ 9,685 and overall costs of amputation due to vascular disease $ 27,987. [2,3] A large percentage of these wounds occur in the growing elderly population. [4] Data from the United States indicates that spending on wound care is likely to grow by about 12% annually.[5] In a survey nurses were found to lack confidence in the knowledge of physicians treating chronic wounds, highlighting the need to standardize the evaluation of wounds. Local statistics about the problem are lacking. In South Africa, a small preliminary cross sectional study concluded the lack of knowledge and education when it comes to the management of chronic wounds. [6] The population demographics and the disease burden bare a closer resemblance to the United States than we would like to admit.[7] Our patients are manifesting with the same cutaneous wound pathologies, and by understanding, examining and deconstructing the chronic cutaneous wound, we will be able to better treat this entity.

Inflammation and biofilm are two common threads in chronic wounds. The mediation of these factors may help in furthering the treatment of these wounds in a more affordable manner. The advent of molecular techniques has enabled the identification of specific targets perpetuating chronic wounds. These advances have brought about a change in dressing and treatment strategy, from debridement and aiding the innate host response, to modifying the

host response in a selective fashion and targeting specific aetiological agents such as biofilm.

This project aims to evaluate the effects of using topical “active” agents with ingredients targeting specific factors of chronic wounds. In a clinical setting where a large percentage of the chronic wound burden is treated in a conservative non-operative outpatient setting, the testing of compounds that can aid wound healing is important. Most patients present with significant co-morbidities, the cost of theatre time worldwide is exorbitant and hence financially less viable; problems that additively oblige further research into the domain of topical wound healing, placing the ingredients where they matter most, at the wound site.

Literature Review

Chronic Wounds

Chronic wounds have been defined and redefined. Current definitions include clinical observation outlining that if a wound is not 30% closed at 4 weeks then it will not close or heal by 12 weeks and this is then defined as chronic.[8] The clinical definitions have been compared concurrently with what transpires at a wound level. Exaggerated inflammation and biofilm are 2 factors that have been identified as perpetuating chronic wounds.[9] They are inter-related factors such that a persistent bacterial load causes an influx of inflammatory cells, cytokines and free oxygen radicals that have a detrimental effect on the ECM bed.

The current principals of treatment of cutaneous wounds follow on the work done by Sibbald *et al* in 2000. [10] They defined three components of local wound care, namely; "debridement, wound friendly moist interactive dressings and bacterial balance". On these principals the concept of wound bed preparation was conceived and the TIME[11] acronym developed. Debridement of non-viable tissue, addressing infection, moisture balance and epithelial edge assessment were the basic strategies of this systematized approach to wound care. Once a wound bed is optimized, the requisite balanced cellular proliferation from fibroblasts, keratinocytes and endothelial cells can progress to a closed wound. The wound bed biochemical and enzymatic makeup has consequently also been delineated in greater detail such that matrix metalloproteinase levels, and other macromolecules that bind growth factors have been implicated in impeding the healing process. (Interleukin 1 (IL1), tumour necrosis factor alpha (TNF -α), interleukin 6 (IL6).[10]

The direction of research then shifted to the wound edge and the cellular changes that were

impeding the final closure of a cutaneous wound by means of epithelial migration. *Falanga et al.*[12] showed that epithelial cells were replicating but not migrating across the wound bed despite what seemed adequate preparation. Many theories have been put forward, including cellular senescence, increased apoptosis and failure of differentiation.

The TIME classification allowed assessment of components of a chronic wound and standardized wound evaluation, allowing more targeted approaches for treatments. The E in the acronym changed from "epithelium non advancing" to "edge of wound" because it was found to be that lack of epithelialisation had as much to do with poor granulation tissue as it does with epithelial cell issues. [12]

The host immune response may hold the solution to the chronic wound problem. The same common pathway results in non-healing wounds. The aetiologies are all varied. This would indicate that tackling inflammation is just as important as addressing biofilm or any other causation in allowing a wound to heal. An imbalance of the inflammatory response is the difference between healed wound and chronic wound. In a clinical context, the systemic wellbeing of the patient is also influential in the healing of wounds, a fact that is easily forgotten when focusing on an ulcer or a constant depth fermenter instrument that grows biofilm in a lab.

Biofilm

Bacteria have been known to cause acute infections from systemic spread of their planktonic forms and secondary mechanisms such as endotoxins. Research over the past 20 years has

proven that bacterial growth modalities exist further than the ones seen in liquid growth media where individual colonies proliferate in a planktonic form. Biofilms are a modality of growth of bacterial species found in the majority of clinical chronic wound situations.[13] Bacterial cells attached to a surface, usually in multispecies colonies in a "slime" substance known as extra-polymeric substance (EPS); EPS being a glycocalyx composed of proteins, lipids, extracellular DNA, alginates and polysaccharides, this is a biofilm. Given time (from 48 hours to mature biofilms in 2 weeks) most bacteria will form a biofilm environment on a wound surface and exist in a sessile state. These can be seen to cause a host of pathologies in clinical situations; chronic respiratory diseases such as cystic fibrosis are perpetuated by the constant presence of bacteria in the alveolar lining; tooth caries become problematic due to the presence of normal microbial flora that become abnormally attached to the damaged lining surfaces of teeth; intravenous catheters and implants become persistently infected due to bacteria which adhere to them in the form of biofilms.

Biofilm cycles can be divided into the attachment, growth or maturation and detachment phases. It is important to understand these phases when researching the effect of biofilms on wounds and particularly when considering treatment strategies. The attachment of bacteria to a surface involves physical and electrostatic interactions with adhesion molecules. The up-regulation of these proteins causes increased binding to collagen, fibrin and fibrinogen (substances making up part of the extracellular matrix).[14,15] Once the bacteria have bound they also undergo a change in expressivity. The genetic makeup of the bacteria resembles phenotypically different structures more aligned to bacteria in a biofilm, than those in planktonic form.

The growth phase of the biofilm results in bacteria communicating and forming stable

populations within a highly structured microenvironment that allows optimum use of nutrients from the wound fluid at the same time evading external measures to eradicate the bacteria. Light microscopy can be used to demonstrate the presence of a matrix (glycocalyx) as early as 5 hours after a wound has been infected.

The final phase is detachment, in which replicating bacteria on the outer surface of the biofilm are released in their planktonic state in order to propagate elsewhere, although the association with causation of invasive disease is not certain.

Table 1 : Factors and the mechanisms that give biofilms an advantage in a wound [16]

Factor	Mechanism
Less Susceptibility to Host Immune Response	❖ Antiphagocytic properties of biofilm, ❖ Most actively replicating bacteria on outer part of biofilm complex ❖ Less active bacteria away from the periphery are protected ❖ Polysaccharides of biofilm block specific complement activation ❖ Bacteria in the inner biofilm are spared ❖ Macrophage activity limited due to biofilm adhesion
Antibiotic Resistance	❖ Inability of antibiotic penetration into the biofilm ❖ Altered microenvironement results in inactivation of antibiotics ❖ Decreased growth rates of bacteria ❖ Quorum sensing – communication between bacteria (inter and intra – species)
Increased Virulence	❖ Gene transfer between different species
Physiological adaptation	❖ Decreased oxygen levels within deeper biofilm ❖ Decreased oxidative stress response ❖ Enzymatic tolerance to heat/cold stress

The persistence of such pathogens in a steady state does however incite a mild but chronic inflammatory state within the context of a clinical wound or affected area; resulting in

chemokines, cytokines and immune cells migrating to the wound site and continuing a degradative process. This degradation is driven by matrix metalloproteinases, persistently elevated levels of inflammatory markers (interleukins, tumour necrosis factor alpha (TNF-α)), inhibiting wound closure by breaking down and inhibiting the formation of collagen extracellular matrix (ECM). Without the sequential build up of ECM (composed of glycosaminoglycans, collagen and proteoglycans), the key processes of cellular migration and epithelialisation cannot take place.

What transpires is a build up of epithelial cells on the edges of the open wound, much like cliff faces descending directly to a sea shore. In a normally healing wound the metaphorical histological pattern is that of sandy beach that gradually goes out to the sea with no cellular build up at a critically hindered point.

Detecting and isolating biofilms and the bacterial species that make up these biofilms represents a logistical challenge. James *et al* showed good clinical evidence that chronic wounds (30 of 50) are far more likely to have biofilm than acute wounds (1 of 16).[13] Davis *et al* demonstrated that biofilm structures could be seen within 48 hours in an acutely infected wound in-vivo. They used a combination of light microscopy, electron microscopy and epiflourescence microscopy to subjectively demonstrate the biofilm on the partial thickness wounds in pigs. [17] The model at 5 days showed higher logarithmic counts of bacteria in wounds that were allowed to form a biofilm as opposed to those wounds that were treated while the bacteria were still in planktonic phase. There is little evidence to show accurate quantification of biofilm on wounds; and therefore measuring in-vitro and in-vivo biofilm eradication has relied on indirect measurements of bacterial culture or by molecular techniques such as peptide nucleic acid fluorescent *in situ* hybridization (FISH) with confocal

laser scanning microscopy.[18] Standard cultures by swab and tissue culture techniques do not isolate bacteria in a biofilm. Antibiotics eradicate planktonic state bacteria whilst those in the inner biofilm are protected. These newer molecular techniques, although promising, are costly and involve highly specialized testing. Semi-quantitative cultures can still be used as a standard of measurement for assessing bacterial burden in clinical wounds.[19] Semi-quantitative cultures also represent a predictive index for wound sepsis/infection. Wound infection was defined as CFU's/g > 10^5 (colony forming units/gram of tissue).[19, 20] Bacterial counts less than 10^5 were deemed to be colonized but not infected. Once a wound has bacterial growth, there is a spectrum of clinical events that take place. All of these events are not detrimental to the patient as a whole. There is however a critical point at which intervention is required. The stages of increasing bacterial burden in a clinical situation are as follows:

- Contamination
- Colonisation
- Critical Colonisation
- Infection

Clinically, these stages have been described by signs at the wound site as defined by *Sibbald et al.*[21]. A correlation between bacterial burden and clinical signs allows for interpretation of semi-quantitative results.

Staphylococcus aureus and *Pseudomonas aeruginosa* are two of the more common bacteria known to form biofilm sessile states in wounds that become infected.[16] They pose a significant clinical problem particularly in burn wounds, as well as the spectrum of chronic wounds mentioned (pressure sores, venous ulcers and diabetic ulcers). These bacteria can also co-

exist within the same biofilm and therefore further compound the treatment problem.

The current strategies being proposed for the treatment of biofilm in clinical wounds involves multi-approach concurrent therapies utilising physical/mechanical debridement coupled with chemical eradication. The multi-dimensional approach aims to decrease the bacterial load and aid the immune system in achieving a balance in favour of a healing wound trajectory. Surgical debridement forms an important pillar of the treatment strategy as it not only breaks up existing mucous barriers but also exposes the bacteria to the host's immune system in a direct manner. Hydrosurgery and radiofrequency ablation are 2 newer forms of surgical manipulation. Hydrosurgery has been particularly used in burns and radiofrequency has been shown to have some efficacy in healing wounds faster than controls. Many differing forms of surgical debridement are available. Not all patients are always candidates for surgical therapy due to co-morbidities preventing the use of general or regional anaesthesia and the prohibitive costs of theatre visits.

Chemical debridement has also been tested in clinical and in-vitro settings. Anti-microbials are controversial in their efficacy against established biofilms as the penetration of these antibiotics to the core bacteria is minimal. Used as an adjunct to surgical debridement though, the antibiotics are useful in controlling the spread of bacteria prohibiting attachment to other surfaces. Antiseptics (biocides such as chlorhexidine, hydrogen peroxide) have been widely described but are required in concentrations 1000 times greater than what they would be to kill planktonic bacteria. Additionally they have a potentially detrimental effect on healing as they are also cytotoxic. Many potential anti-biofilm agents have been tested both clinically and in-vitro with little success. Immunotherapy, vaccine therapy and targeting quorum-sensing genes all have shown promise in the in-vitro environment but in in-vivo testing lack

the same efficacy due to interactions with wound mediators and proteins.[22]

BF (Flavonix®) has reported in-vitro inhibitory activity against *S aureus* and *P aeruginosa*, and importantly also has an anti-biofilm potential causing breakdown of the polysaccharide barrier. NCS (Acticoat®) has similar demonstrated anti-microbial effects, but relatively less anti-biofilm effect when used alone[23].

Treatments: Flavonix® and Acticoat®

A non-healing chronic wound is caused by an imbalance of inflammatory mediators as a result of a persistent biofilm and bacterial burden. If the mediator balance can be maintained such that the extracellular matrix is not broken down; this should aid a wound to heal by a more pronounced epithelial response and migration across the matrix bed.

Both BF (Flavonix®) and NCS (Acticoat®) have the potential to control the exaggerated inflammatory response as well as affect the biofilm in a wound. These "active" dressings represent an advance in wound healing management; modifying and modulating factors within the wound rather than just mopping up exudates or creating moist healing environments in which the wound was allowed to heal. With a greater understanding of the causes of poor wound healing, these products allow a more targeted approach to healing, bearing in mind the important systemic management principals in any wound.

NCS (Acticoat®) has long been considered an effective treatment and preventative dressing for cutaneous wounds. Its anti-bacterial properties as well as anti-inflammatory tendencies have made it a popular choice of dressing in the treatment of recalcitrant wounds as well as acute wounds[24,25]. Its anti-inflammatory effects on the wound bed have been well described.

[24] This effect is a consequence of its inhibitory action on matrix metalloproteinase (MMP) enzymes (specifically MMP-9, gelatinase B), thereby making it an ideal dressing for chronic wounds with exaggerated inflammation and typically a higher concentration of MMP-9.

BF (Flavonix®) is a relatively new formulation, containing many naturally occurring constituents, which have been tested separately to address inflammation, bacterial infection and importantly biofilm. Clinical case studies have been reported using this product on chronic wounds; yet the outcomes of these observer-based studies do not allow an insight into the wound milieu[26]. No study has been carried out on this product evaluating specific criteria. The collection of ingredients making up the Flavonix® gel target biofilm breakdown and suppression of inflammation within a wound. The individual products have been tested in isolation on various models. Table 2 highlights the key ingredients in the formulation and their potential influence on the wound.

Table 2: Active ingredients found within Flavonix® gel with potential effects on wound healing.

Active Ingredient	Potential Effect on Wound	Reference
Baicalein	Anti-inflammatory, anti-oxidant	27
Oleuropein	Anti-inflammatory, anti-bacterial	28,29
Vitamin E	Anti-oxidant	30
Farnesol	Anti-biofilm – dissolves fibrin fibres	31
Xylitol	Anti-biofilm- inhibits Glycocalyx formation	31
Aloe Vera	Anti-inflammatory, anti-bacterial	32
Witch Hazel	Anti-inflammatory	33
Honey	Anti-bacterial	34

The use of such products in the treatment of chronic/stalled/recalcitrant wounds will be increasingly common, given that the disease burden is increasing. Newer simpler tests being investigated allow wounds to be evaluated from a biochemical perspective, thereby

indentifying specific factors/mediators requiring correcting. Although the use of specific factors (e.g. growth factors) promises much, studies have shown equivocal results due to the complex interactions of many mediators within a wound. The use of active targeted dressings provides a new approach to addressing non-healing wounds.

Background to choosing an animal model

The study of cutaneous wound healing relies on application of cellular and biochemical knowledge to the clinical situation. In-vitro studies are well suited to examining one or two targets in isolation; indeed newer synthetic scaffolds have made it possible to more closely mimic the skin. However, the complexity of cutaneous healing (in particular chronic wounds) and its multiple interactions between host, bacteria, cells, mediators and indeed the practicalities of dressing the wounds, warrants the use of in vivo animal models in order to more realistically evaluate the effects of a specified treatment modality. The goal of animal models for wound healing studies is to create an analogous situation to humans; where pathological processes result in chronic non-healing wounds, allowing duplication of the complexities of that wound and the various interactions that take place within the wound. This is not possible with in-vitro analysis as these models regulate all but the tested parameters allowing little simulation of paracrine and autocrine mechanisms.

Although existing animal models of chronic wounds include a variety of animal species as well as pressure models, ischaemia models, and burn models[35,36,37]; the challenge is to find what works within a particular environmental context. Pressure models and ischaemia models require specialized devices and are logistically labour intensive. Burn models have been traditionally described in larger animals like pigs where the morbidity per burns surface area

was less than with smaller mammals like rats. In a study conducted by Gurfinkel[38] burns of 5 x 5 cm from a non contact heat source at 400° C for 20 seconds resulted in 30 – 50 % mortality of rats depending on their size. Therefore careful burn creation is required for survival of the animals.

Porcine models incur less morbidity due to their ability to handle a greater insult of wounds; yet result in far higher cost per animal for housing and technical challenges in repeatedly taking animals for procedures and dressing changes etc. Small mammals such as rats are easier to handle and cheaper to keep. Also the number of animals required to create an adequate sample size (n = 6-8) does not make the porcine model a feasible one. An initial Flavonix® safety study was conducted using a porcine model (Female Large White Pigs). 3 animals were used in this study and the study duration kept relatively brief. The results of this study are seen in Appendix A (unpublished data).

The advantage of the porcine model is that it mimics human skin healing, where wound epithelialisation is the primary mode of healing as opposed to wound contraction. [39] In loose skinned mammals with a panniculosus carnosus layer (rats, rabbits, mice etc) the principal mechanism of healing is wound contraction. The drawbacks in using the rat model are adequately dealt with using modifications such as the splinting of wounds to negate contraction as a factor.[41] Table 4 tabulates some differences in skin architecture between human skin and loose skinned mammals.

Table 4 : Major differences between human skin and skin of loose-skinned mammals like rats[35]

	Loose skinned Mammals	*Humans*
Epithelial Architecture	No rete ridges	Rete ridges
Biomechanical Properties	Loose, compliant and thin	Thick, adherent and stiff
Subcutaneous muscle layer	Panniculosus carnosus throughout	Only present in the neck as the platysma muscle
Major method of healing	Wound contraction	Granulation and epithelialisation

Materials and Methods

This chapter outlines the animal model, products used and tests undertaken to acquire histological and microbiological data to evaluate the early wound healing events in an in-vivo chronic wound treated with Acticoat® and Flavonix®. In brief, a modified burn excisional wound model replicating a chronic wound was used in Sprague-Dawley rats, with four groups, two treatment and two control groups being assigned for evaluation of early wound healing events

Wound Dressing Details

Acticoat® (Smith and Nephew, London, UK)

10cm x 10cm sheets of Acticoat® (Smith and Nephew, London, UK) were used. These were cut into discs of 2cm diameter each in order to fit the wounds. The discs were soaked in water for approximately 10 min prior to application onto the wounds as recommended by the manufacturer. A secondary Postop Opsite® (Smith and Nephew, London, UK); a clear

polyurethane adhesive square with a central absorptive pad; was placed over this in order to hold the Acticoat® in place.

Flavonix® (Medika, South Africa)

Aliquots of 1ml of gel were used directly onto the wound at each dressing change. The gel was covered with a secondary dressing of Postop Opsite® (Smith and Nephew, London, UK).

Bacterial Inocula Formation

The ATCC strains used were control strains from the Department of Clinical Microbiology and Infectious Diseases : *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213.

The Lubbock Model protocol as described by Sun *et al* (2008)[40] was used to create an adequate bacterial laden broth used to inoculate the excised burn wound bed on postoperative day 4 (POD 4).

Bolton broth (Oxoid, South Africa) (see Appendix B) with 50% heparinised bovine plasma (V/V) and 5% freeze-thaw laked horse red blood cells (V/V), was used as the growth medium. 10 µl of a normalized culture ($1x10^6$ CFU/ml= 0.5 McFarland Standard) was added by injecting the pipette tip into the broth, thereby serving as the surface for biofilm formation. Inoculations included a combination of Pseudomonas aeruginosa ATCC 27853 and Staphylococcus aureus ATCC 29213 (5 µl of 0.5 McFarland of *P. aeruginosa* and *S. aureus* respectively). These were then incubated at 37^0C with shaking (140*rpm*) for 24 hours.

To make the broth, 13.8 grams of the powder was dissolved in 500 ml of water and then autoclaved. The aliquots of broth were then done when making up the final medium which consisted of 3ml of heparinised bovine plasma (Onderstepoort, Faculty of Veterinary Sciences, University of Pretoria), 2.3 ml of Bolton broth (Oxoid, South Africa) and 0.7 ml of laked horse blood (Oxoid, South Africa) as per the protocol. 0.1ml of broth was inoculated to all wounds with a sterile measuring pipette at POD 4.

Animal Unit Wound Protocol

Female Sprague – Dawley rats (+- 300g) were housed and conditioned initially in groups of 5 in a cage, a week prior to commencing surgery. The animals were housed in separate cages with food and water *ad libitum* 48 hours prior to surgery. All animal husbandry procedures were followed according to the Central Animal Services Unit (University of the Witwatersrand, Johannesburg) protocol. The facility maintained a controlled environment with 12 hour day and night cycles. All operative procedures were carried out in the Central Animal Services Laboratory, 1st Floor, University of the Witwatersrand, Medical School Campus, Parktown. The study was approved by the University of the Witwatersrand Animal Ethics Committee (Ethics approval number : 2010/48/04).

Anaesthetic, Resuscitation and Euthanasia Protocol

Preoperatively, meloxicam 1mg/kg *po* (non-steroidal anti-inflammatory dissolved in berry flavoured jelly) was given 12 hours prior to surgery, with the rats being habituated to the placebo jelly for 4 days prior to surgery. Morphine sulphate 10mg/kg was given *sc* 20-30 min prior to surgery. Intraoperatively isofor 5% was used to induce anaesthesia and 2% isofor was used for maintenance via mask delivery. All animals were operated on a heated blanket

and transferred to a heated blanket postoperatively. Immediately postoperatively, recovery was maintained on a heated blanket between 24° C and 26° C. Five millilitre boluses (ringers lactate with dextrose 5%) were given intraperitoneally (*ip*) at body temperature as a resuscitation bolus. Morphine sulphate 10mg/kg *sc* was given 3 hours post operatively and bupranorphine 0.02 mg/kg *sc* 3 hours after morphine sulphate for analgesia.

Euthanasia on Days 10, 14 and 21 was performed with a single dose of Pentobarbitol 100mg/kg *ip*.

Wound Creation and Batching Protocol

A total of 128 female Sprague-Dawley rats (±300g) were used. The rats were divided into 6 batches of 16 rats/batch. Within each batch of 16 rats, 12 rats were inoculated with a bacterial broth and 4 were not inoculated at all (no broth). All animals were treated with the same secondary covering dressing protocol, consisting of Postop Opsite® (Smith and Nephew, UK, London), and three other reinforcing layers. Negative control animals in each batch were of two types. Inoculated negative controls that were inoculated with broth but not treated with any "active" agent and non-inoculated negative controls where no inoculum was placed and no "active" treatments were placed either. Of the eight remaining animals per batch, four were treated with 1ml of Flavonix® Gel and four with 2cm water moistened discs of Acticoat®. The initial operative day was taken as Day 0, the day on which the burn was created. Each batch of 16 rats was begun on a separate date, such that no two batches were run concurrently, but rather in a staggered fashion. Batches were limited to 16 rats in each as this was the maximum number of rats that could safely be done in one session without encountering unplanned complications and subsequent deaths of the animals postoperatively,

especially after the initial burn at Day 0. Animals were housed in separate cages from POD 0. An aseptic technique was used to handle each animal at the time of the surgical procedures and at termination. A sterile technique was not adopted, as this does not replicate a normal dressing change in a clinical situation. The daily handling of animals during cage cleaning and food rationing was done without any aseptic technique being used.

Burn Protocol at Day 0

Animals were anaesthetized as described, and placed on a drape. All animals were shaved around the area to be burnt. A brass block measuring 2x2cm (weight 80g) was heated in hot water (96 °C) for 20 secs. This heated block was then placed on the dorsum of the rats back approximately 2cm from the scapula edge in a para-midline position while the skin was held taught by hand. (Figure 1). The block was allowed to stand on the skin surface for 10 seconds and then removed. The weight of the block ensured a uniform force on each burn wound. The rat was then treated as per resuscitation protocol and returned to its individual cage. Monitoring was performed every 2 hours for the first 6 hours; recording animal behaviour according to a standardised assessment protocol used by the Central Animal Services Unit. Any animal found to be in undue distress was assessed for possible euthanasia and a joint decision with the veterinarian was made to this end. These assessments were then performed twice daily for the duration of the study.

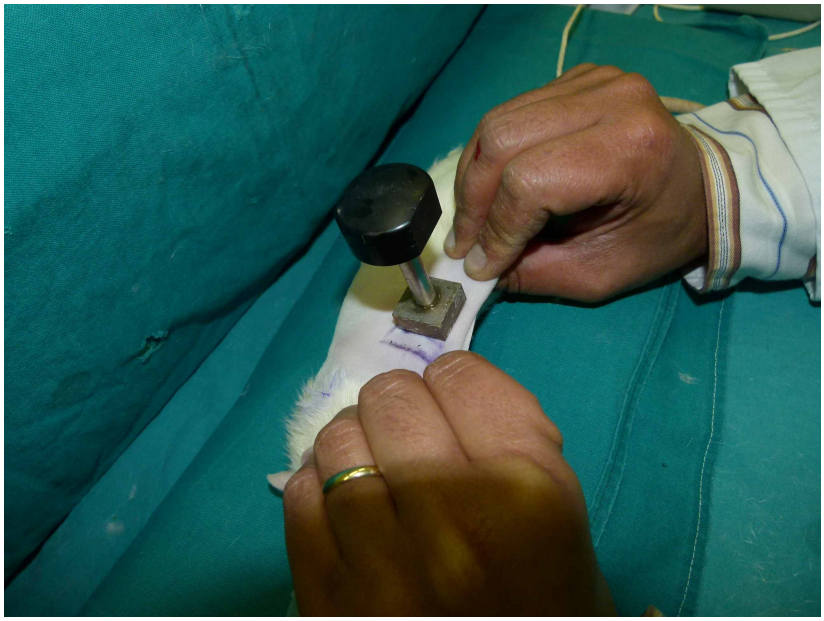


Figure 1 : Placement of the 2 x 2 cm brass block placed for a period of 10 secs on skin that was tented out to get an even contact.

Burn Eschar Excision, Splinting and Inoculation at Day 4 (POD 4)

On POD 4, the burn wound was excised in a circular fashion with a no. 15 scalpel blade (Swann Morton®) using the tracing of a 2cm diameter silicone rubber ring (Bearing Mann). The entire burn eschar including the panniculosus carnosus was excised and a full thickness wound of 2cm diameter created. The silicone ring was then placed around the wound and fixed with adhesive glue (cyanoacrylate) and a running 4/0 nylon (Ethicon, South Africa) suture. (Figure 4) Once the wounds were excised and splinted, 10µl of the bacterial broth (0,5 MacFarland Standard Concentration 1 x 10^6 CFU's/ml) was injected onto the wound site with a sterile measuring pipette. The wounds were left for a further 3 days till POD 7 before treatment was commenced with the "active" topical products. This period of 72 hours allowed a biofilm to establish within the wound.

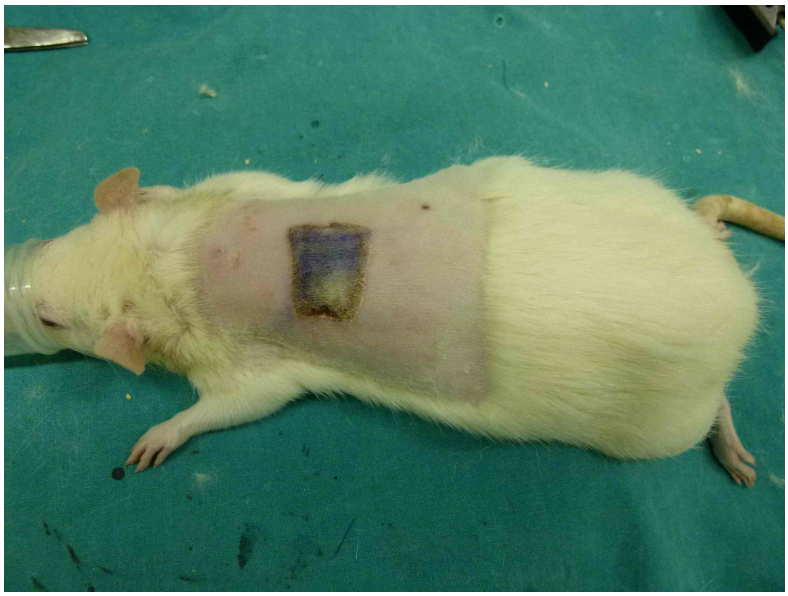


Figure 2 : Wound at Day 4; full thickness burn eschar to be excised and splinted

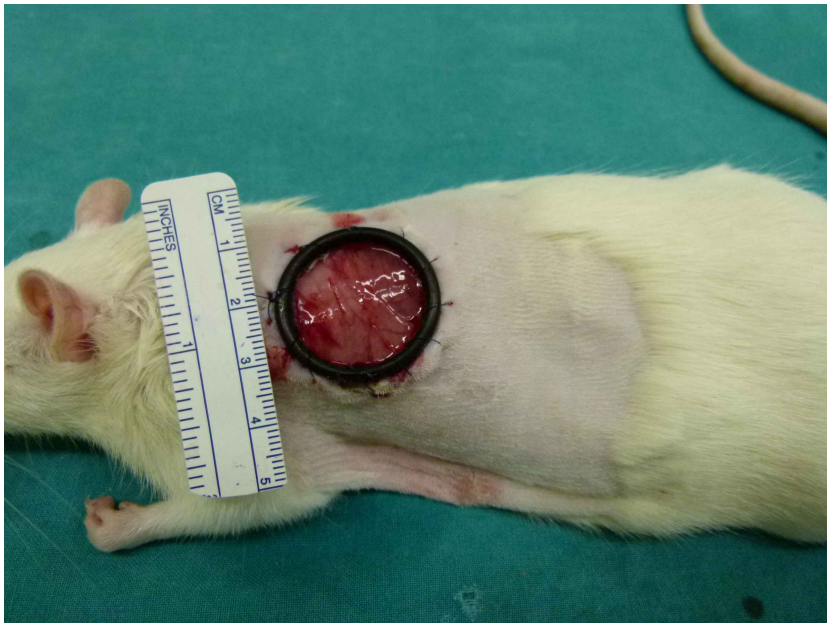


Figure 4 : Wound at Day 4 after full thickness excision (including panniculus carnosus) of eschar and application of silicone rubber ring to splint the wound with adhesive glue and running nylon suture

Dressing Protocol

Dressings were changed on days 10, 14, 17/18 and 21. In those animals that required "active" topical dressings, an Acticoat® 2cm diameter disc presoaked in sterile water was placed in the

Acticoat® cohort and 1ml of Flavonix® gel was injected onto the wound using a 5ml syringe in the Flavonix® cohort. The wounds were then covered with a Postop Opsite® (Smith and Nephew, UK, London) adhesive dressing. A further 3 protective secondary dressing layers served as additional protective outer layers to prevent the rat from biting through to the wound. The first of these was a 25 mm stretchable tape (Leukoplast Elastic, BSN Medical), the second a self contact stretchable bandage (Vetcrepe, Kyron Labs) and the final layer a 25mm zinc oxide tape (Healthease, SA). The outer protective layers did not prevent free movement of the animals and did not restrict breathing. (Figure 4) Four additional rats in one of the Day 14 cohorts had Flavonix® and Acticoat® applied onto the wounds at POD 7, and 10. The moist Acticoat® was applied over the 1ml of Flavonix® gel that was placed first onto the wound. The rest of the dressings for these additional rats remained exactly the same.

The rats were then treated as described in the "Burn Protocol at Day 0" and returned to individual cages. Two hourly checks documenting behaviour and feeding were carried out for the first 6 hours. Thereafter twice daily checks were carried out. Any interference by the rats of their dressings was addressed at the next available time for a dressing change.

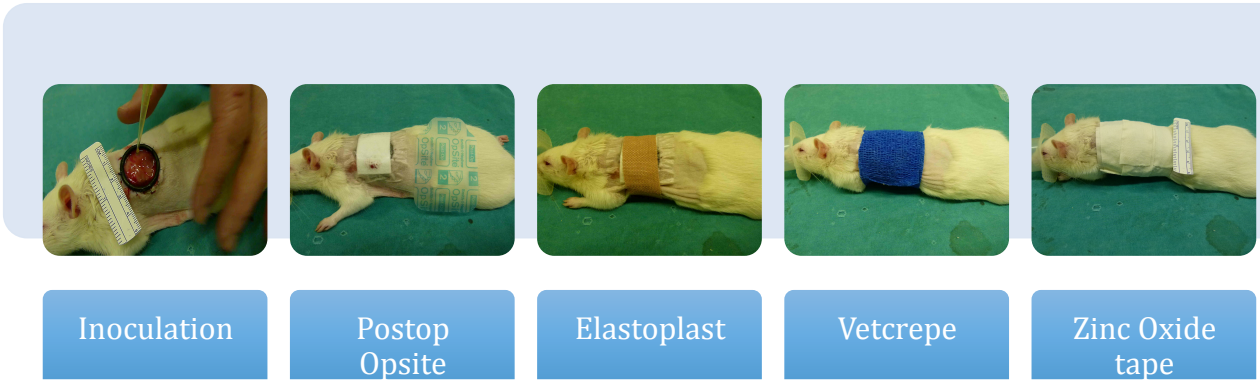


Figure 4 : Wound Dressing Protocol - from Inoculation to Final Dressing on POD 4

Termination of Animals at Determined Time Intervals and Treatment Group Assignment within Batches

Terminations of batches were carried out the at following time points : Day 10, 14, and 21 days postoperatively, the operative day (POD 0) being the day on which the burn was created. Batches were assigned with 16 rats in each. Two batches would be terminated at each of the stipulated days. Within each batch of 16 animals, 4 were assigned to each of the treatment groups (n=8 at each time interval); Flavonix®, Acticoat®, Negative Control (NC) and a Non-inoculated Control (NIC) Group. On Day 14, four additional rats were terminated. These rats had been treated with a combination Acticoat® and Flavonix® gel as described in the "dressing protocol". None of these animals were included in the results due to the insufficient sample size of this group (n = 4). The outcomes in these animals is discussed briefly in the "discussion" section of this report. The non-inoculated control group gave an indication of the healing potential of a non-infected burn wound over 21 days. (Figure 6) The 21 day period includes part of the proliferative phase of wound healing.

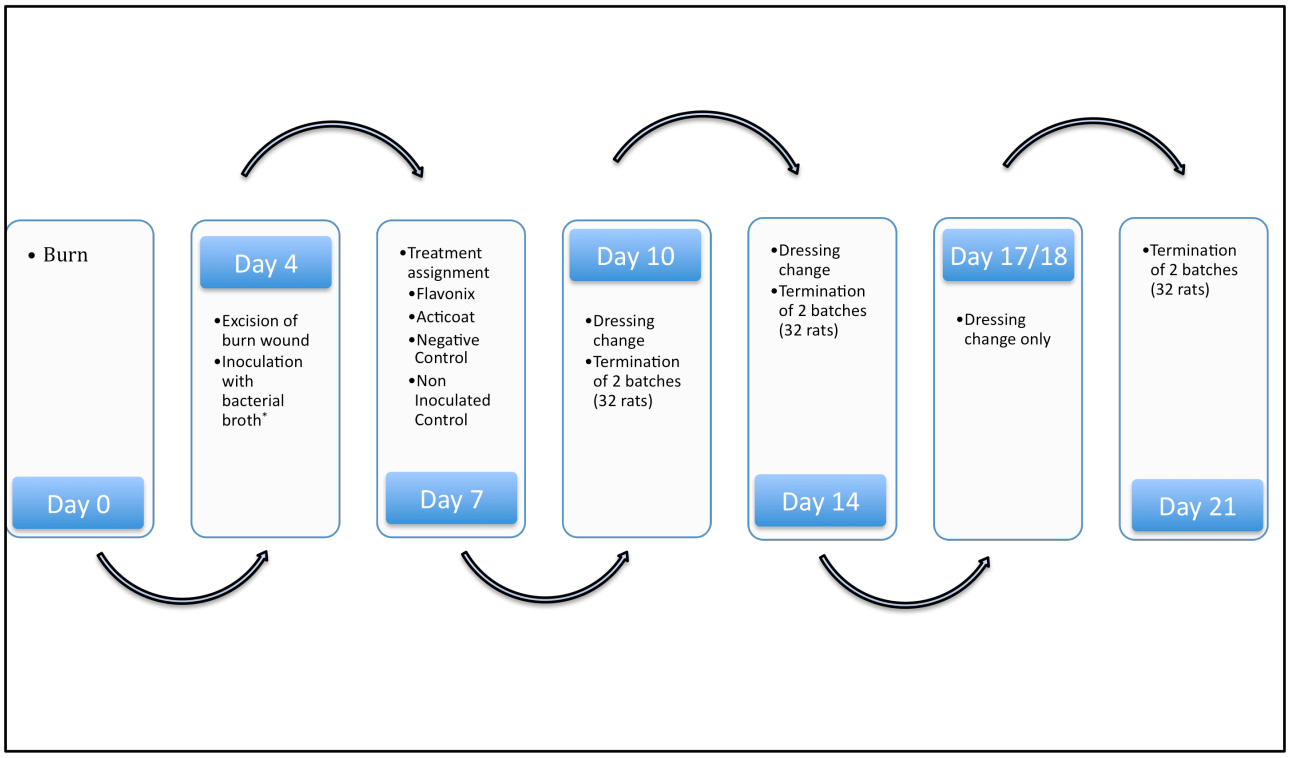


Figure 6 : Timeline showing Days and Events at each timepoint

Wound Site Preparation, and biopsy allocation for histology and semi-quantitative culture.

On termination, the wound sites were washed and superficial debris removed by the use of a hydro-debridement process. The silicone splint ring was removed and the nylon suture excised. The wound was then irrigated, using a funnel (an inverted 20ml syringe) into which 20ml of normal saline was injected with a 20ml syringe via a 20G intravenous cannula creating a high enough pressure to remove superficial debris. Between wounds all disposables (eg. gauze, surgical blades) were discarded and all re-usable instruments (forceps, stainless steel scissors) disinfected by rinsing in a solution.

The wound site was completely excised with a 5mm perimeter of normal tissue. The excision was full thickness including the muscle under the granulation bed. The excision was performed with a no. 15 surgical blade (Swann Morton, UK). The specimen was placed on a cleaned flat sectioning board.

The tissue was then halved down the centre, creating 2 equal semi-circular specimens. One half was then inked on its undersurface (Rotring Drawing Ink™ and a histological tissue fixative (Cancer Diagnostics Inc™)) for identification of the deep surface and the specimen placed on a solid surface for preparation of biopsy samples for histology, immunohistochemistry. At the widest diameter of the semicircular piece of tissue a plastic ruler flattened the tissue and a 4mm strip of tissue was sectioned using a no. 10 stainless steel surgical blade (Swann Morton, UK). This strip represented the greatest diameter of the wound (20mm). Each strip was pinned to a cardboard strip with straight pins for orientation purposes with the inked side indicating the deep surface of the wound. The inked, pinned specimens were then placed in containers with a 10% buffered formalin solution. These were sent to Lancet Laboratories (Johannesburg) on the same day for processing and evaluation. (Figure 8).

The other semi-circular portion of sectioned tissue was placed in a sterile container for semi-quantitative culture.

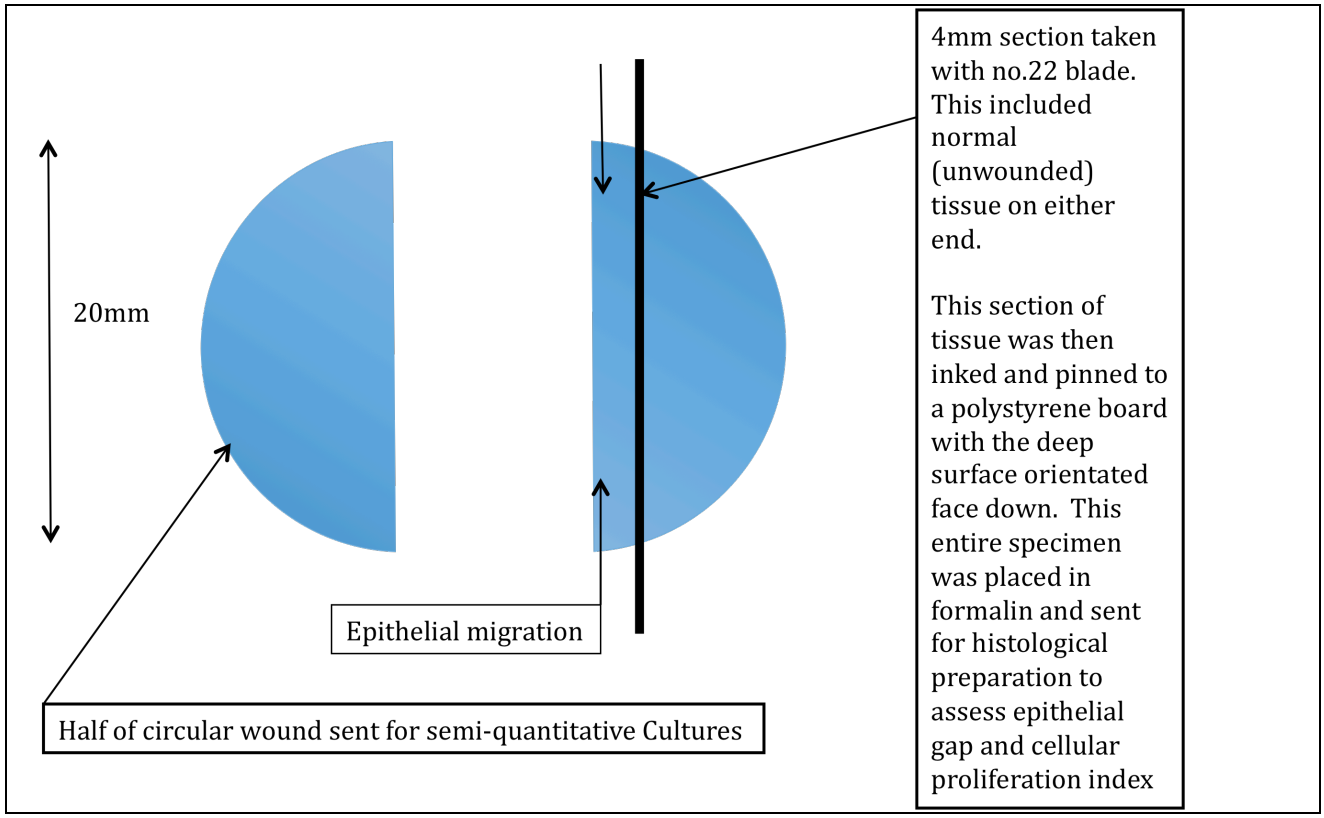


Figure 8 : Schematic diagram showing processing of the wound specimen for histology and semi-quantitative culture.

Histology and Epithelial Gap Measurements

After an initial macro processing, the specimen was orientated according to the deep margin which had been inked and longitudinal slices were taken and placed into a cassette for impregnation in a Sakura®Tissue Tek Vacuum Processor and Embedder. The process involves washes with formalin, methanol (70 – 100%), xylene and finally wax. This was done as per the Lancet Laboratory Protocol.

Sectioning of the tissue was conducted on a Leica® microtome with 2-3µm slices being taken and then mounted onto slides by melting wax in water baths at 48˚C followed by further heating at 60˚C.

Staining of the formalin fixed, paraffin embedded sections involved initial Haemotoxylin (nuclei) and Eosin (cytoplasm) staining using a Tissue Tek Prisma (Sakura®) Processor. The dehydration and hydration cycle used xylene and methanol and then water immersion prior to haemotoxylin staining. This was followed by immersion in Scott's solution and further rinsing in eosin, ending with repeat washes in water, methanol and xylene before a cover slip was applied.

The H&E stained slides were used for measuring the epithelial gap distance in specimens. (Figure 10) This was done using light microscopy (Olympus BX51) at 10X magnification. The epithelial edges of the wound on each slide were identified and marked with a super fine felt tipped pen (Pentel NMF 50), then removed off the stage and measured with a Vernier's caliper (TA 670500) to the closest millimeter. Measurements of the epithelial gap were performed at POD 10,14 and 21 for both the treatment and control groups. The use of a measuring graticule on the microscope stage to measure the epithelial gap was not possible due to the inability to fit the entire length of each slide onto the microscope stage at the lowest magnification (10X magnification). The markings were therefore done microscopically and the measurement done macroscopically using a Vernier's caliper off the microscope stage. The inaccuracy of this method should be noted and was mitigated by taking measurements to the nearest millimetre.

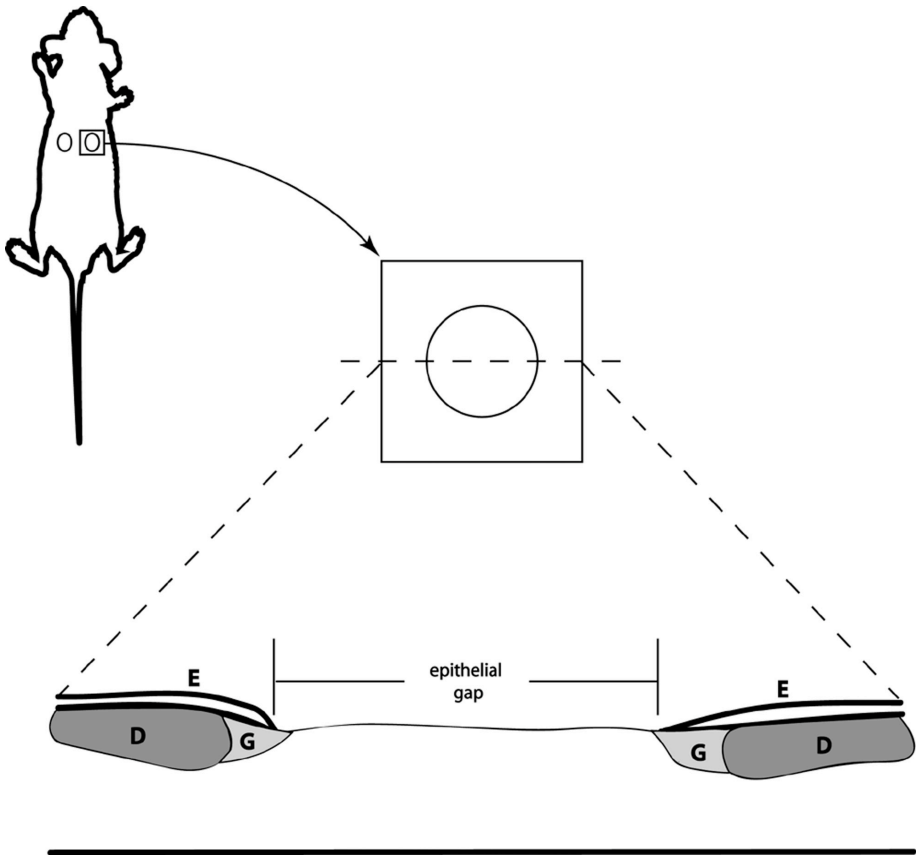


Figure 10 :Schematic representation of the epithelial gap measurement. (Taken from Galiano *et al.* Wound Rep Regen 2004. [41]) Epithelial Gap was measured using Vernier's caliper measurements after marking the epithelial edges using light microscopy at 10X magnification (Olympus BX51). Epithelial gap measurements were taken to the closest millimeter. E = advancing epithelium, G = granulation tissue, D = native dermis.

Immunohistochemistry: Ki-67

Immunohistochemistry was performed, utilizing Ki-67 nuclear antigen as an immunolabelling target to assess the relative amounts of replicating basal cells in the sections prepared. The slide sections used for measurement of Ki-67 labelling were the same sections as for epithelial gap measurement that had undergone the process outlined below.

Ki-67 allows detection of cells in the active phases of the cell cycles (G phases). In normal wound healing the epidermal cells behind the advancing cell edge start to replicate and epithelial migration is facilitated by hemi-desmosomal intercellular links breaking between

epidermis and basement membrane.[42] Manual image analysis was chosen to evaluate the proliferation index within each wound as cost constraints and lack of available digital analysis facilities prohibited digital analysis. Manual analysis has the following drawbacks; subjectivity, observer variability and time expense.[43] Methods of image analysis described include scanning slide specimens and then using computational analysis to detect stained proportions. Without the available equipment and also standardized calibrations, this option was not available to us.

The Dako Envision™ FLEX Detection System used on an Autostainer Link instrument gives optimal staining when used as per protocol. This staining was carried out at Lancet Laboratories (Johannesburg).

Pretreatment Procedure

Paraffin-embedded tissue sections fixed in formalin were used. Sections were prepared on FLEX IHC Microscope Slides (code K8020). The slides were mounted from a preheated water bath containing distilled or de-ionised water. Sections were dried by heating at temperature of not more than 60˚ to ensure proper adherence of the sections to the slides as per protocol.

A 3-in-1 specimen preparation procedure using PT Link as described in the Envision™ FLEX Kit (Dako) manual was used. Deparafinnisation, rehydration and Heat Induced Epitope Retrieval (HIER) was performed with this protocol, using DakoCytomation Target Retrieval Solution, Citrate pH 6, code No 2369.

Immunolabelling Procedure

Immunolabelling involved the use of Dako FLEX Ready-to-Use Primary Antibodies (Anti-Rat Ki-67 Antigen MIB-5 Clone, code no M7248) at a concentration 2ug/ml (113/ug/ml with a dilution ratio of 1:50) with the Dako Envision® FLEX Detection system. The immunolabelling steps and incubation times were preprogrammed into the Autostainer Link (Dako). The control antibody was DakoCytomation Mouse IgG1 (code No X0931) diluted to the same concentration as the primary antibody. Haemotoxylin (blue) was used as the counter-stain. The diaminobenzidine(DAB)-containing substrate working-solution (Envision ™ FLEX) gives off a brown colour when the secondary antibody binds the primary antibody at the recognized antigenic site activating the chromogenic reaction (staining) between substrate and horse radish peroxidase (HRP).

Each slide had an outermost section of tissue composed of a full thickness unwounded skin. This section of skin was used as a "normal" frame of reference, by which to compare the immunolabelling in the wounded skin. The comparison was based on morphological findings and relates to the cellular proliferation index which was determined as explained in the text further. The target antibodies and the secondary antibodies were tested on normal (unwounded) rat skin samples from unwounded animals. Positive controls were assessed using the target antibody (Anti-Rat Ki-67 Antigen MIB-5 Clone, code no M7248) and negative controls using a DakoCytomation Mouse IgG1 (code No X0931). The specificity of the primary antibody was assessed morphologically comparing staining in the stratum basale to the staining in the progressively superficial layers of the epidermis as well as to other background areas in the tissue section (dermis). Positive staining in the stratum basale was expected

morphologically to be greater than in other background areas (dermis and more superficial epidermal layers). The secondary antibody control was tested without the use of a primary antibody and assessed morphologically also in normal rat skin (unwounded animals).

Cellular Proliferation Index Determination

A single observer method was used to assess all slides morphologically. In order to mitigate against the pitfalls of visual assessment the following criteria were followed. Fields were distinctly outlined so that high power field (HPF) allocation was not random and variability hence minimized. Five sequential HPF's on each side of the wound beginning at the epithelial margin were analysed for positively immunolabelled chromogen stained cells. Only cells positively immunolabelled in the stratum basale were counted for the numerator of the ratio (Figure 12). The denominator of the ratio was formed by the sum of the positively immunolabelled cells and the cells stained by haemotoxylin in which nuclei were observed. This is expressed further in this text as the "total number of cells" in the stratum basale (total number of cells = number of positively immunolabelled cells + number of haemotoxylin stained cells in which nuclei are identified). The observer and cross sectional variability of this method has been noted as a potential flaw of this method due to some nuclei not being included as a result of the cross section. A ratio of stained (positively immunolabelled) over total number of cells in the stratum basale (as described) was calculated for each HPF initially. Corresponding HPF's on either side of the wound were averaged. Cell counts for each HPF were then further divided by a baseline cell count in a normal skin HPF for each section (Figure 14, Figure 16). This baseline cell count was taken as the ratio of stained (positively immunolabelled) cells/total number of cells in the HPF most lateral on the slide section. The ratio in this HPF section would most resemble normal unwounded skin. A final relative ratio was then calculated by dividing the ratio of each HPF (1 to 5) over the ratio of the baseline

HPF (Figure 16). This ratio is then referred to as the cellular proliferative index. The closer to 1 the index, the closer the resemblance toward normal skin. Each HPF measured 450 µm in length.

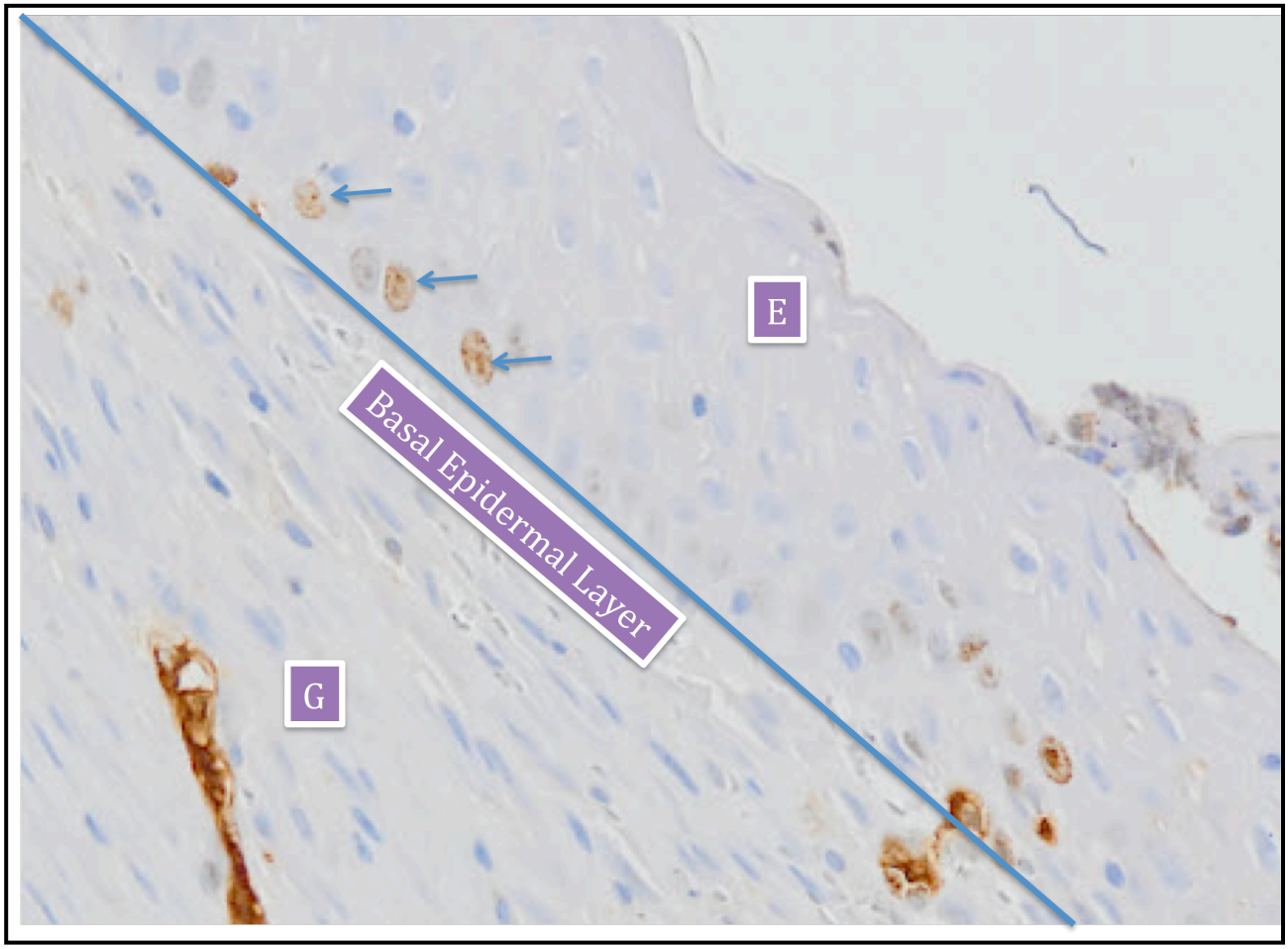


Figure 12 : Cross sectional view of healing wound at 20X magnification showing the granulation bed (G) on which the epithelial migrating layer (E) is advancing. The arrows indicate the positively stained Ki-67 cells in the basal epidermal layer marked by the blue line. The Ki-67 immunolabelled cells staining positive cells with the background haemotoxylin (blue) stain highlighting the nuclei of nonproliferating cells. Note only those cells in the stratum basale were counted. The ratio is therefore the number of positively immunolabelled cells in the stratum basale /total number of cells in stratum basale in a HPF. Total number of cells = Immunolabelled cells + haemotoxylin stained cells in which nuclei were positively identified.

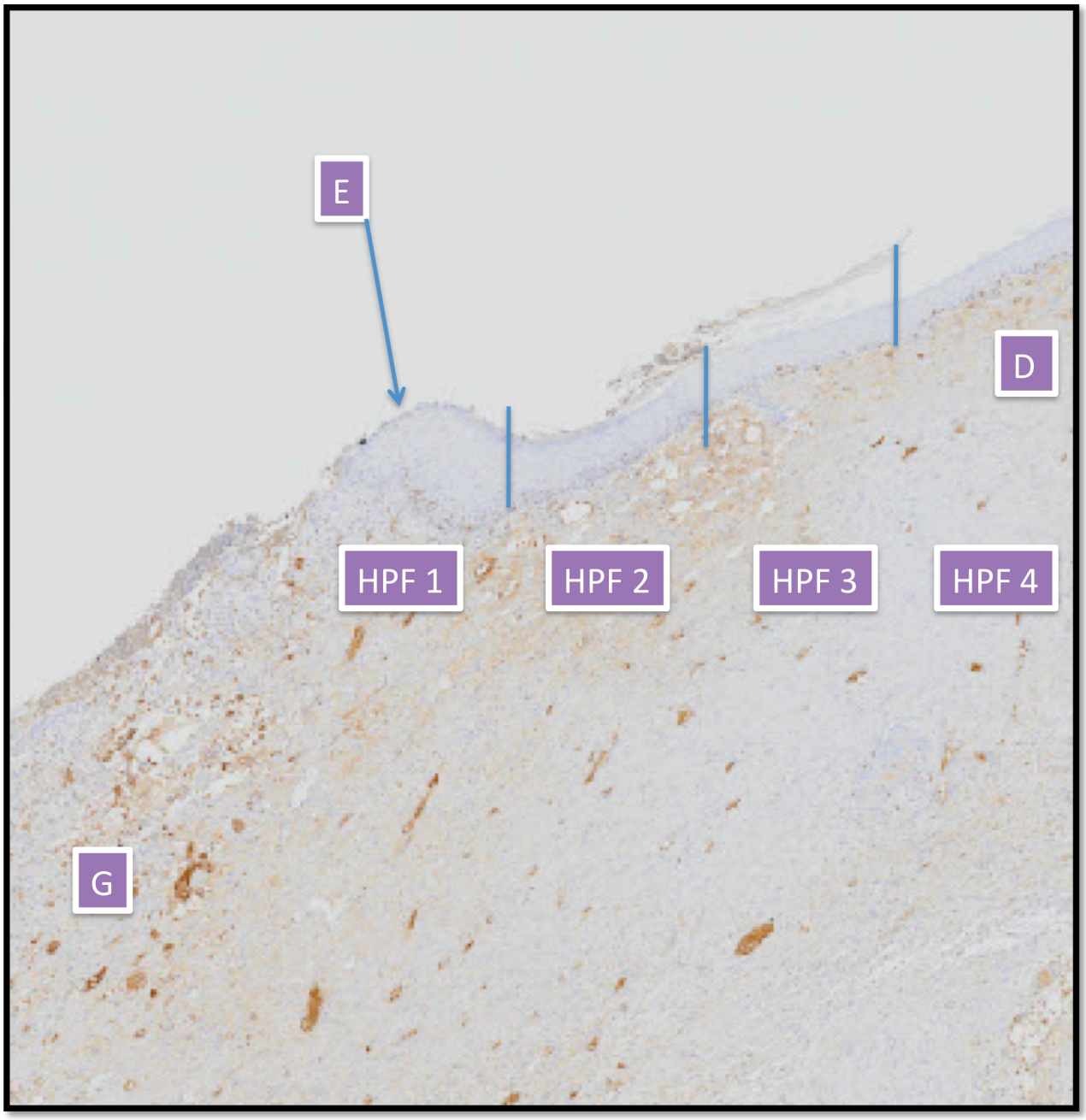


Figure 14 : Histological slide (10X magnification) of the edge of a wound showing the advancing epithelium (E), on the new granulation bed (G) with the unwounded dermis (D) at the edge of the wound. The line bars indicate how the HPF's were selected sequentially from the wound edge moving laterally (1 to 5). HPF 5 will be situated lateral to HPF 4, is not seen on this slide.

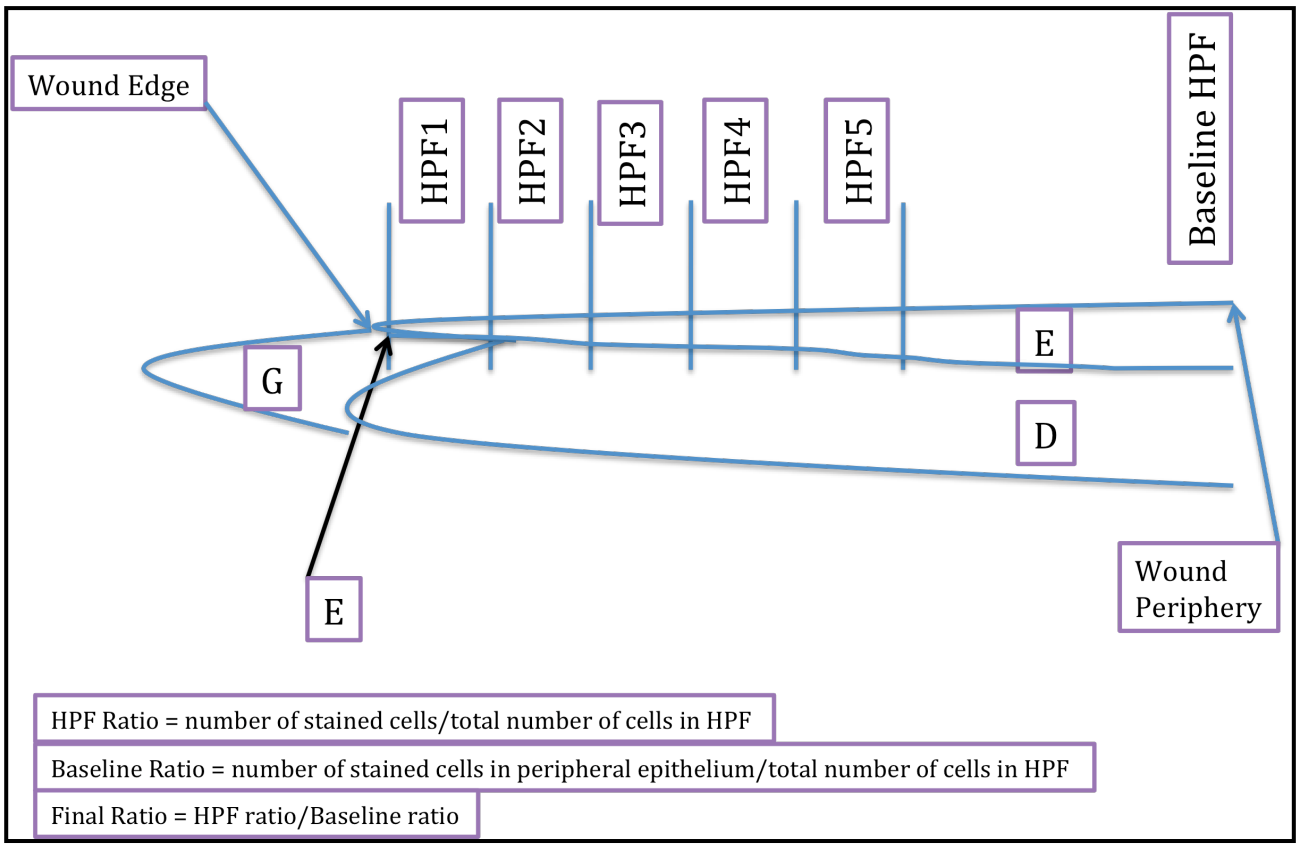


Figure 16 : Schematic diagram of a tissue section showing the advancing epithelial front on newly formed granulation tissue. The diagram indicates the HPF's analysed for the recorded results with Ki-67 staining for cellular proliferation. E = epithelium, G = granulation tissue, D = native dermis. Each HPF field at 40 X magnification is approximately 450 µm.

Evaluation of bacterial load in wounds

Semi-quantitative analysis was performed to get an approximation of bacterial activity within the wound at POD 10 and 21. Clinically significant bacterial counts were set at CFU's/gram tissue > 10^5.[19] This simplifies the assessment of the bacterial load into wounds that are infected and those that are not.

Specimens were received in batches of 16. The specimens were processed as per protocol described by Buchanan *et al*.[19] Briefly, after irrigation to dislodge superficial debris, one half of the wound was excised, weighed and transferred using a sterilized forceps into a sterile specimen bottle. The specimen was transferred to Lancet Laboratories (Johannesburg) within 4-6 hours of collection.

All specimens were processed in a standardized format by technicians who were blinded to the study. Specimens were homogenized in a grinder container with 2ml brain heart infusion broth. Each of two blood agar and two MacConkey plates received 10µl of broth per plate and a further two plates of each type received 100µl broth specimen per plate. Plates were incubated at 35°C and read at 24 hours and 48 hours for *S. aureus (*Pastorex Latex agglutination kit positive (Biorad F92430)) and *P.aeruginosa* (Cytochrome oxidase positive (Prolab Diagnostics, Canada)).

The final results were documented as colony forming units/ gram of tissue. The calculation for CFU's/gram = $N \times D_{sq} \times V/W$,

N = number of colonies on a plate of a given dilution,

D_{sq} = reciprocal of the homogenate volume,

V = volume of diluent used for homogenization

W = weight of tissue in grams.

All tests were conducted in duplicate and the volume of homogenate chosen was done so specifically to give counts of CFU's between 10^4 and 10^6. Only those specimens with CFU's between 10^4 and 10^6 were then counted. The threshold for a clinical wound infection was taken as CFU's > 10^5 /gram tissue. The result of the semi-quantitative culture for each specimen was then reported as positive if the CFU's/gram tissue exceeded 10^5 and negative if the result was less than 10^5, as per protocol by Buchanan *et al*.[19]

Statistical Analysis

Epithelial gap and cellular proliferation data (both continuous variables) were tested for normality using a Shapiro-Wilk normality test. The data was found to be non parametric and therefore a Kruskal-Wallis test was used to detect homogeneity between groups. A Bonferroni post hoc analysis then provided an intergroup comparison with a p value < 0.05 being accepted as significant between groups.

Microbiological data was represented by a categorical variable; data being categorized as a clinically infected wound if CFU's $> 10^5$, and not infected if CFU's $< 10^5$. The data was analysed using a Fisher's exact test comparing each group to every other group. A significance level of $p < 0.05$ was used.

All statistics were done using the statistical package Statistica (ver 10).

Results

The following chapter describes the results of the three data sets, namely epithelial gap, cellular proliferation and semi-quantitative culture findings from POD 10 to POD 21 during the early wound healing phase. Results between groups are compared statistically for significance at each time interval, and also within groups between different time periods (i.e. POD 10 vs. POD 21 for each group).

Epithelial Gap Measurement

The treated groups showed increased epithelialisation rates when compared to the negative control group at POD 21. Table 6 shows the results of the treatment groups versus the control groups. On POD 10 all wounds were of a similar size with the treated groups showing no significant change on the wound closure within the first three days post commencement of treatment. At Day 14 the signs of epithelialisation are evident on the wounds. Those animals in which the splint had been bitten through and was not effective for longer than a day were excluded from the data as the effects of wound contraction then played a significant role in closure. The variation in "n" indicated in Table 4 accounts for those animals in which the splint had been bitten off. A divergence in the wound healing trend is noted at Day 14 (see Figure 17). The Flavonix® (14.5mm ± 0.79) and Acticoat® (14.71mm ± 0.57) groups have a greater wound closure than both the negative control group (16.67mm ± 1.01) and non-inoculated group but this is not significantly different. At Day 21, the Acticoat® group (8mm ± 0.96) has a significantly smaller epithelial gap than the negative control group (12.8mm ± 0,65) . The Flavonix® group (10.33mm ± 1.05) did not show a statistically significant difference to the negative control group. The Flavonix® group showed the same closure to the

non-inoculated group (10.33mm ± 0.99) at POD 21. The trend in closure of these two groups was faster than that of the negative control group but not statistically significant.

Table 6 : Epithelial Gap Results from POD 10, 14 and 21. Mean (mm) ± SEM (N).

Days PO	Acticoat	Flavonix	Negative Control	Non-Inoculated Negative Control
10	18.8 ± 0.39 (10)	18.86 ± 0.31 (14)	18.6 ± 0.58 (10)	19.33 ± 0.28 (12)
14	14.71 ± 0.57 (14)	14.5 ± 0.79 (12)	16.67 ± 1.01 (12)	15.29 ± 0.61 (14)
21	8 ± 0.96 (8)	10.33 ± 1.05 (12)	12.8 ± 0.65 (10)	10.33 ± 0.99 (12)

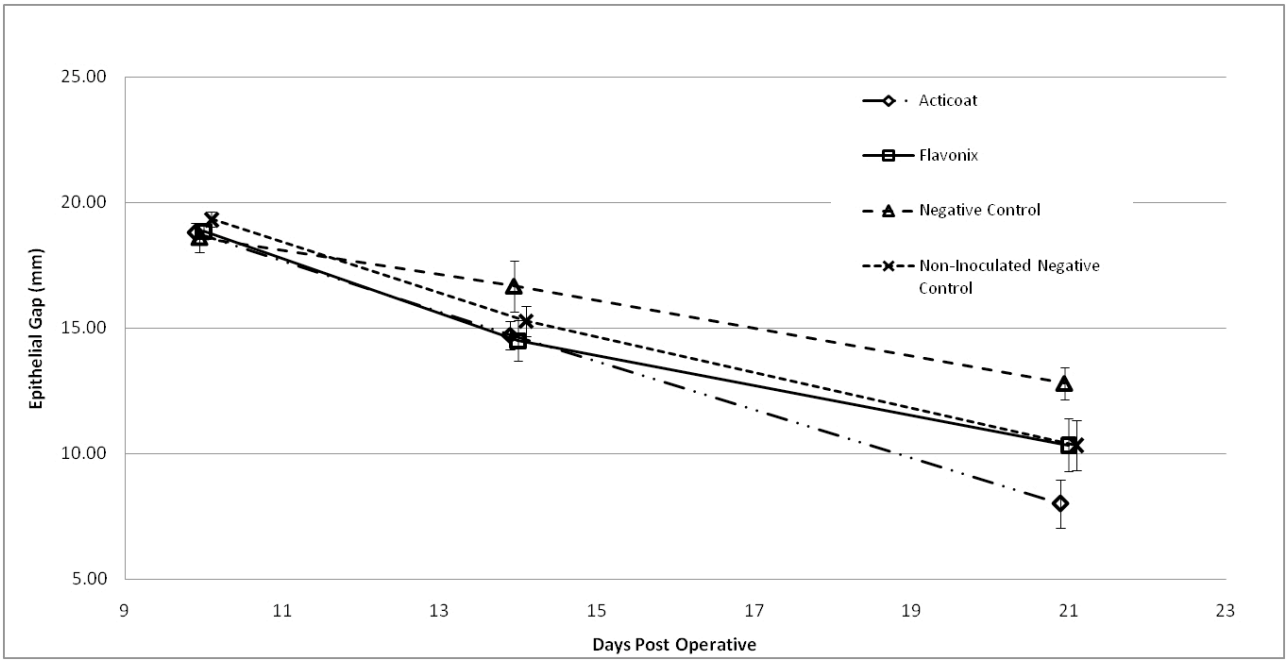


Figure 17 : Graphical representation of the epithelial gap data. Days 0-9 were excluded as the wound was created and inoculated at Day 4, treatment commenced at Day 7 and the first measurements carried out at Day 10. The largest size of the wound was 20mm on Day 7. The trends of wound closure of both treatment groups and the non-inoculated control group exceeds that of the negative control group. Only the Acticoat® group (8mm± 0.96) is significantly different at day 21. ($p < 0.05$)

Epithelial gap by itself represented the endpoint of the healed wound. The wound milieu and the changes affected by the treatments needed further evaluation, as both treatment groups were having an effect on the healing wound. These effects were noted clinically by healthier granulation tissue and the extension of epithelium across the wound (Figure 19). The Ki-67 immunohistochemical staining served as an indicator for cellular proliferation and as an indirect measure of inflammation in the healing epithelial front.

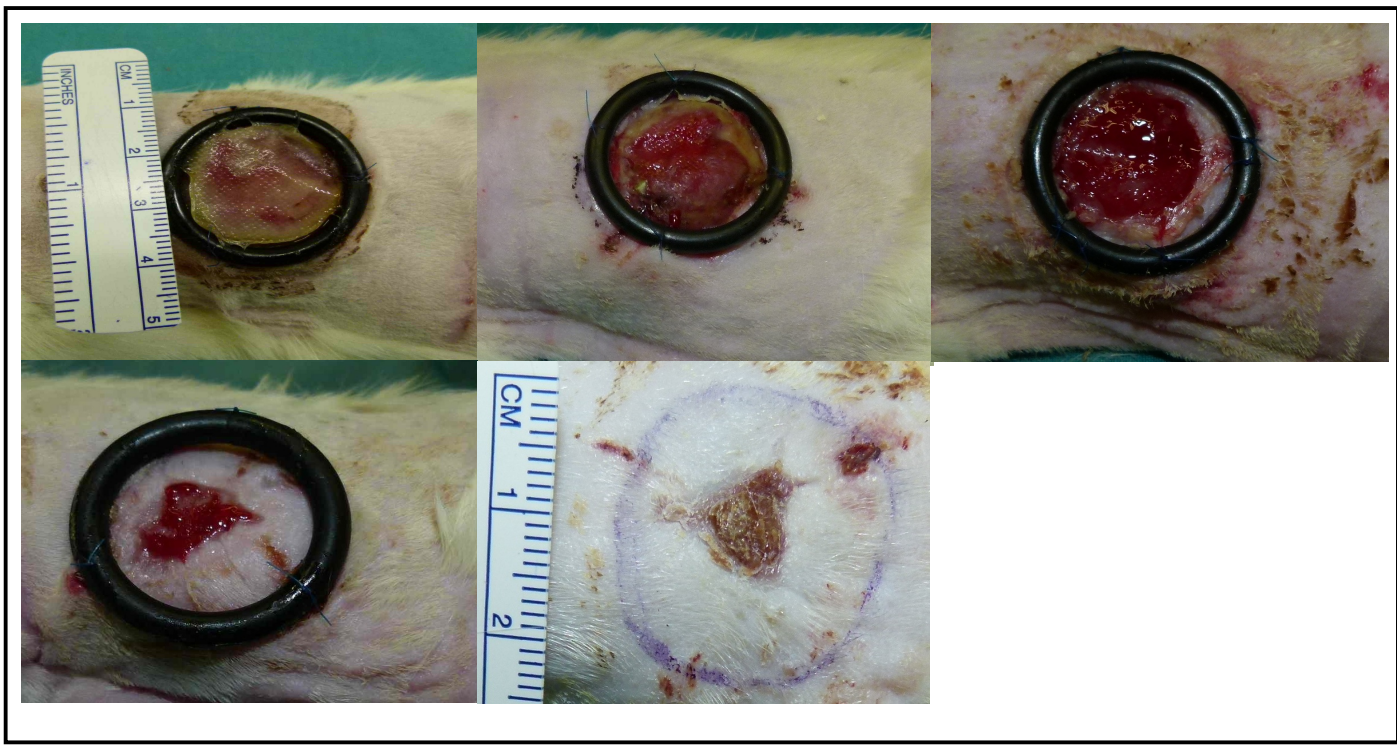


Figure 19 : Sequential healing of a chronic wound in a Sprague-Dawley Rat using the splint method to minimise the effects of wound contraction in healing of the wound. The sloughy and "slimy" layers on the wound on POD 7, 10 and 14 can be seen to be improving not only by closure but also by a better looking granulation bed. No wounds were completely closed at Day 21. The inked area marked the position of ring after it was removed on at termination, and demarcates the original wound size.

Immunohistochemistry: Ki-67 staining/Cellular Proliferative Index

Table 8 : Cellular proliferation index per high power field. Mean, SEM, (N). A ratio of 1 is indicative of normal unwounded skin.

Treatment	Days PO	HPF1/Base Line	HPF2/Base Line	HPF3/Base Line	HPF4/Base Line	HPF5/Base Line
Acticoat	10	1.18 ± 0.6 (6)	4.16 ± 3.19 (6)	4.18 ± 3.34 (6)	1.98 ± 1.49 (6)	1.96 ± 1.81 (6)
	14	0.82 ± 0.23 (14)	1.57 ± 0.31 (14)	1.83 ± 0.33 (14)	1.54 ± 0.28 (14)	1.37 ± 0.36 (14)
	21	4.01 ± 1.96 (4)	4.69 ± 1.59 (4)	5.68 ± 1.85 (4)	2.67 ± 1.11 (4)	1.99 ± 0.88 (4)
Flavonix	10	1.45 ± 0.6 (10)	4.48 ± 1.58 (10)	2.73 ± 0.99 (10)	1.43 ± 0.82 (10)	1.01 ± 0.67 (10)
	14	1.16 ± 0.32 (12)	1.28 ± 0.17 (12)	1.38 ± 0.38 (12)	1.16 ± 0.26 (12)	0.77 ± 0.26 (13)
	21	1.21 ± 0.29 (10)	1.45 ± 0.23 (10)	1.2 ± 0.21 (10)	0.81 ± 0.17 (10)	0.84 ± 0.3 (10)
Negative Control	10	5.65 ± 5.37 (6)	4.2 ± 2.27 (6)	5.09 ± 2.47 (6)	1.49 ± 0.94 (6)	0.88 ± 0.59 (7)
	14	2.48 ± 0.84 (10)	3.1 ± 1.75 (10)	2.05 ± 0.65 (10)	1.43 ± 0.36 (10)	0.97 ± 0.38 (10)
	21	6.2 ± 2.32 (8)	8.65 ± 2.06 (8)	7.93 ± 1.89 (8)	7.94 ± 2.31 (8)	4.6 ± 1.23 (8)
Non-Innoculated Negative Control	10	4.39 ± 1.13 (8)	5.76 ± 2.07 (8)	5.58 ± 3.09 (8)	5.16 ± 2.51 (8)	2.56 ± 0.72 (8)
	14	3.17 ± 0.75 (12)	3.78 ± 1.19 (12)	3.39 ± 0.61 (12)	1.73 ± 0.39 (12)	1.83 ± 0.48 (12)
	21	2.91 ± 1.05 (12)	5.27 ± 2.11 (12)	5.28 ± 2.53 (12)	2.01 ± 0.95 (12)	1.6 ± 0.73 (12)

The cellular proliferation indices indicate that cellular proliferation beginning from the wound edge (HPF1) and extending for five consecutive high power fields (HPF's) away from the wound edge has a typical skewed bell shape. This is demonstrated in all the graphs. (Figure 21,Figure 23,Figure 25). Cellular proliferation of epithelial cells is less at the verge of the healing margin and increases dramatically in the area immediately adjacent this. The proliferation of cells then tapers off gradually toward normal cell proliferation on the edges of the specimen resembling more normal unwounded skin. Normal skin would be taken to have a cellular proliferation index of 1. The variation in "n" is due to only those specimens being included in which 5 HPF's were identified on either side of the wound edge.

At POD 10, (corresponding with six days after the wound was created and three days since the first treatment was administered) the ratios of all groups followed the same skewed bell shaped pattern with proliferative activity of the cells being highest in the high power fields (HPF's) one field away from the wound edge. Each HPF represents 450µm. There is no significant difference in the cellular ratios between groups at this time point (POD 10) in any of the fields (Table 8).

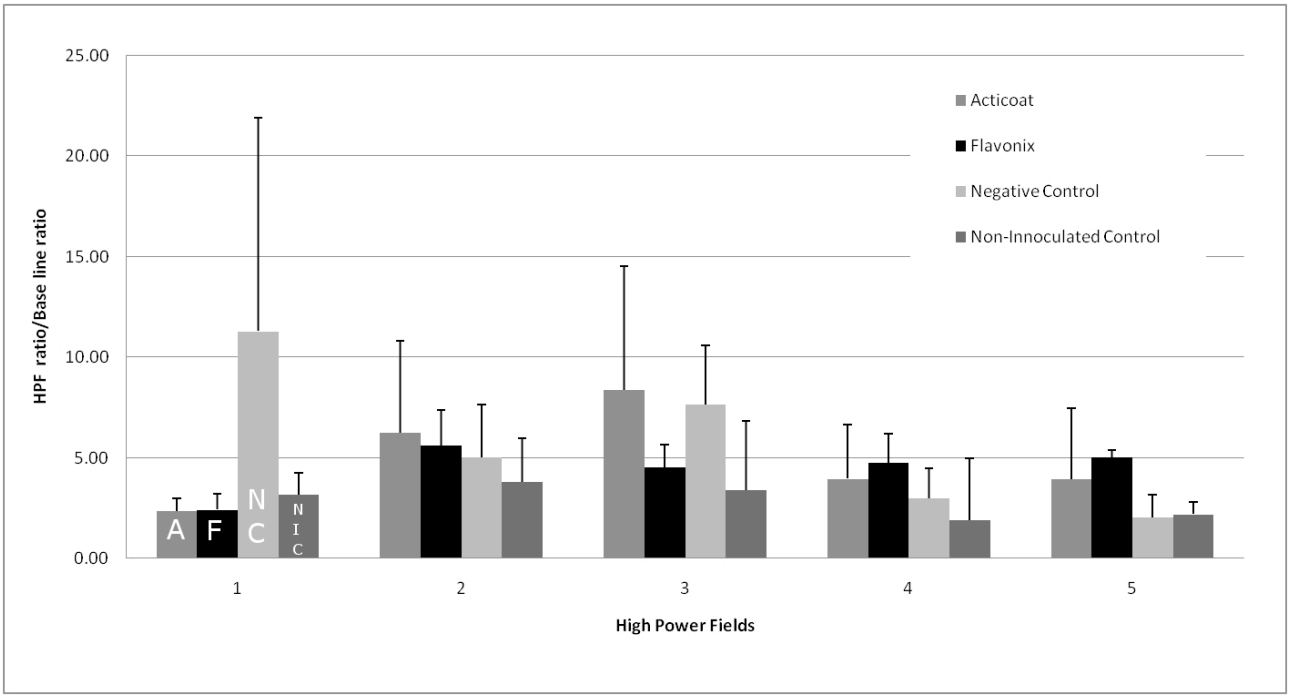


Figure 21 : Day 10 Cellular proliferation ratio graph (mean, std error bars). Five successive HPF's extending from the epithelial edge (HPF 1) towards the periphery of the section (HPF 5). Each HPF measured approximately 450µm. HPF 1 is the field closest to the wound edge and HPF 5 is furthest away.

At POD 14, (corresponding with 10 days after the wound was created and seven days since the first treatment was administered) the cellular proliferation profile looks very similar in general shape. However the two treated groups, Acticoat® and Flavonix® show a reduced cellular proliferation profile compared to the control groups. These profiles were not shown to be statistically significant.

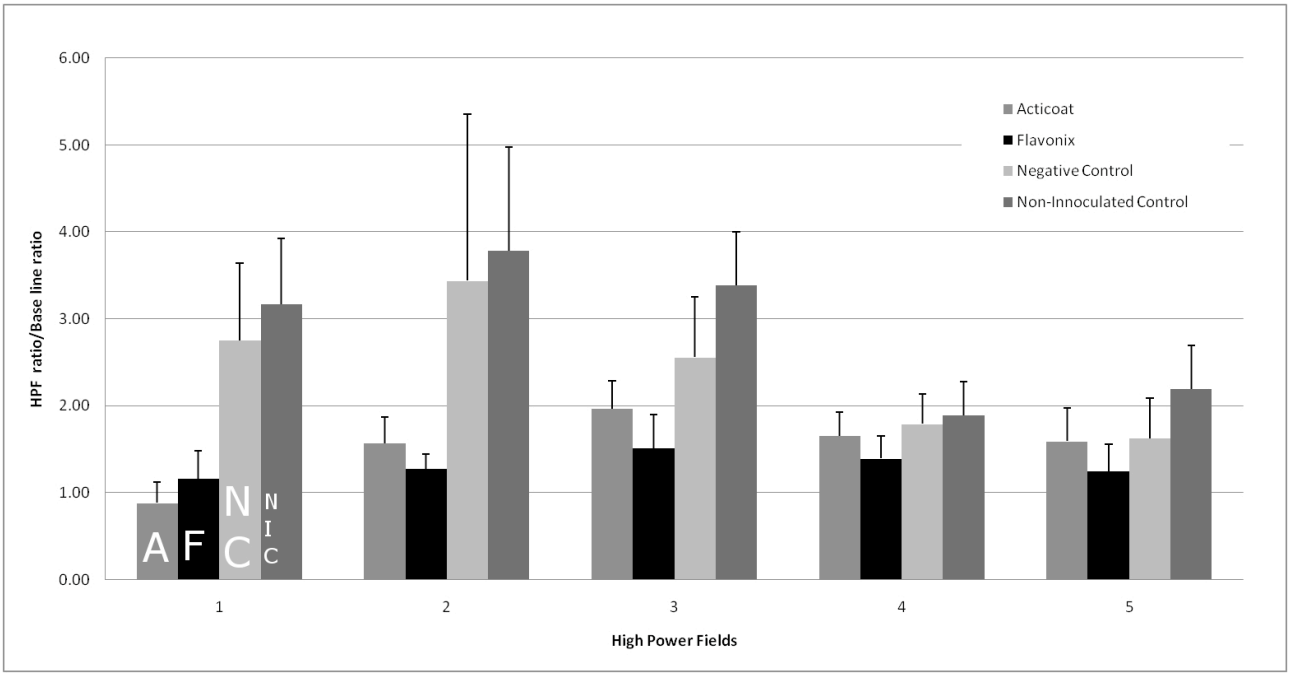


Figure 23 : Day 14 cellular proliferation (mean, std error bars)

At POD 21, (corresponding with 17 days after the wound was created and 14 days after the first treatment was commenced) the proliferation of cells in all groups bar the Flavonix® group has increased two to three times from POD 14 to POD 21. The Flavonix® group has remained suppressed in terms of cellular proliferation. This difference is statistically significant in HPF's 2 and 3 between the negative control groups and Flavonix® group. The proliferation in the Flavonix® group seems to be globally suppressed in all HPF's. The Acticoat® group although showing a lower proliferation than the negative control and the non – inoculated group still shows a near doubling of proliferation when compared with its POD 14 results.

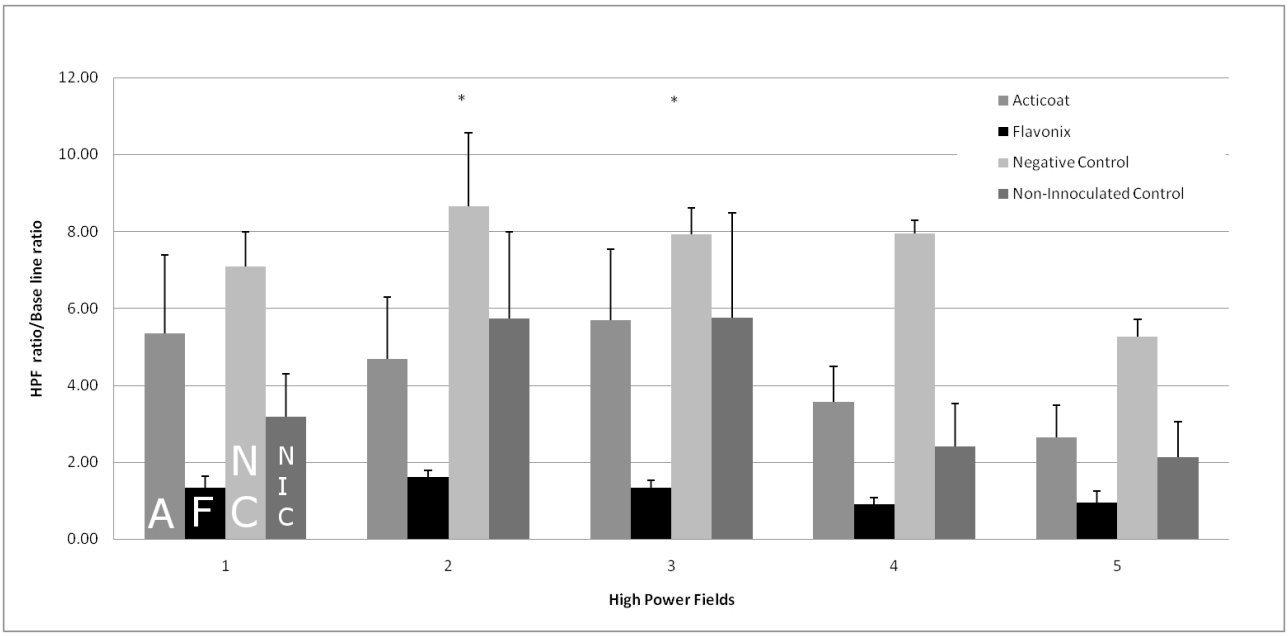


Figure 25 : Day 21 cellular proliferation (mean, std error bars). There is a significant difference between the Flavonix® group and the negative control group in HPF's 2 and 3 is noted (*). Although significance was not observed in all the HPF's of note is the cellular proliferation of the Flavonix® group which is closer to the baseline value of 1 than any other group (black bars). The Acticoat® group shows a less suppressed cellular proliferation profile.

Microbiology

The wounds were analysed by semi-quantitative culture at POD 10 and 21 as per protocol described. Those cultures above the threshold of CFU's > 10^5 were seen as being positive for being an infected wound. The variation in "n" is due to specimens which were not included due to poly-microbial cultures. These were deemed contaminated.

At POD 10, the majority of samples showed bacterial levels above the clinical threshold (CFU's > 10^5). In all treatment groups collectively *P. aeruginosa* and *S. aureus* species were present, 87.5-100% and 50-87.5% respectively. There was no significant difference between any groups at this time.

Considering *P. aeruginosa* at POD 21, Flavonix® treatment showed a reduction below clinical threshold levels in 100% of samples (Figure 27) . Compared to Day 10 there is a significant difference ($p < 0.05$) in all groups except the Acticoat® group. (Table 9)

Considering *S. aureus* at POD 21, none of the groups showed a significant reduction in clinical threshold levels (Figure 29), when compared to Day 10. There was also no difference between groups on POD 21. (Table 11)

Pseudomonas aeruginosa (% CFU's > 10^5)				
Days PO	**Acticoat**	**Flavonix**	**Negative Control**	**Non-Inoculated Negative Control**
10	100 (7)	87.5 (8)	100 (8)	100 (7)
21	50 (4)	0 (8)	50 (8)	42.86 (7)
Data presented as percentage of plates with CFU's > 10^5 (sample size)				

Table 9 : Semiquantitative analysis for *P. aeruginosa* at Days 10 and 21. (% CFU's ,(N))

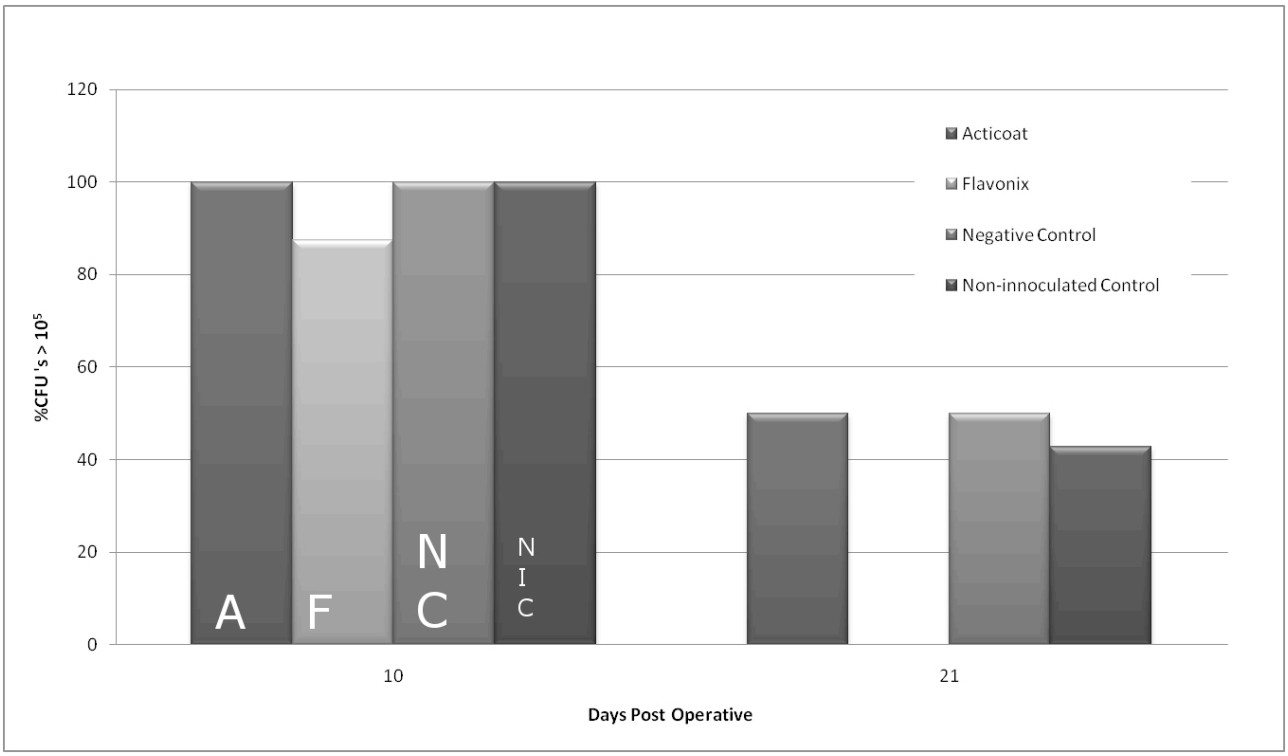


Figure 27 : Day 10 and 21 data showing the percentages of wounds above clinically infected threshold (>10^5 CFU/gram tissue) with *pseudomonas aeruginosa* as determined by semi-quantitative culture.

Staphylococcus aureus (% CFU's > 10^5)				
Days PO	**Acticoat**	**Flavonix**	**Negative Control**	**Non-Inoculated Negative Control**
10	85.71 (7)	87.50 (8)	50.00 (8)	85.71 (7)
21	50.00 (6)	75.00 (8)	87.50 (8)	71.43 (7)
Data presented as percentage of plates with CFU's > 10^5 (sample size)				

Table 11 : Semiquantitative analysis for *S. aureus* at Days 10 and 21. (%CFU's, (N))

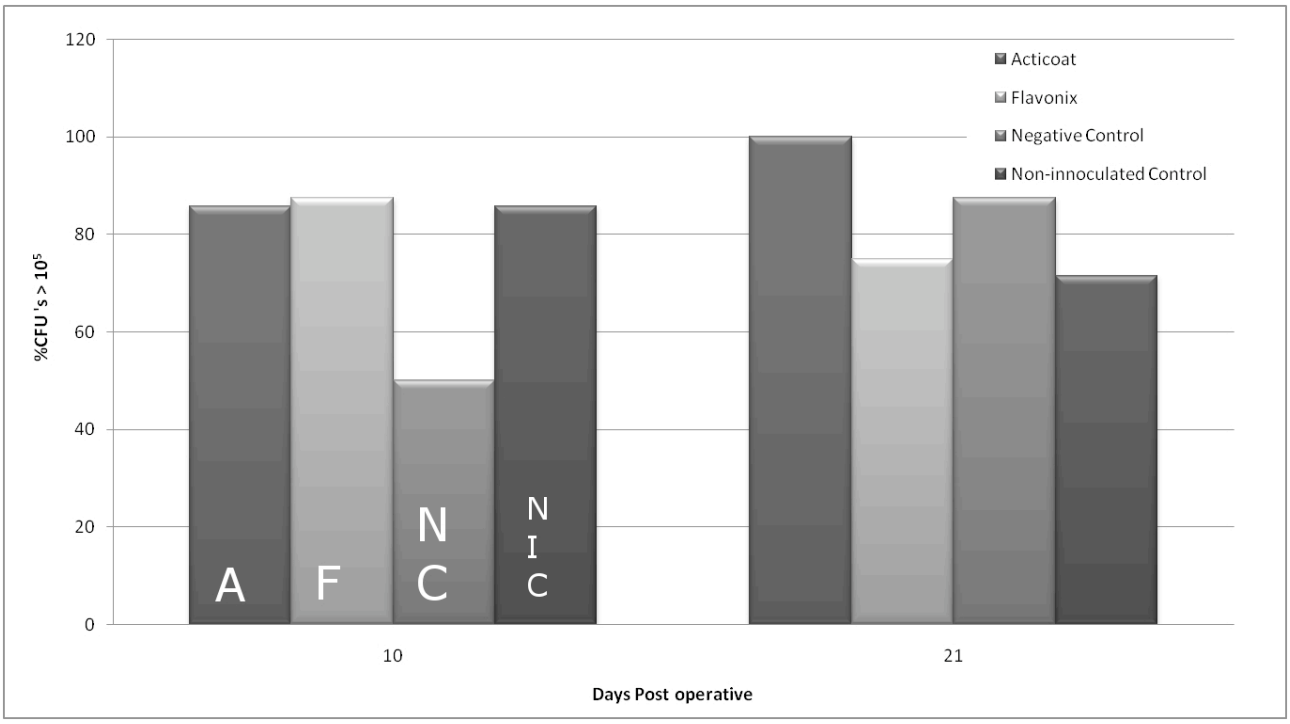


Figure 29 : Day 10 and 21 data showing the percentage of wounds clinically infected (>10^5 CFU/gram tissue) with *staphylococcus aureus* as determined by semi-quantitative culture.

Discussion

This study has shown some of the effects of a BF (Flavonix®) and NCS (Acticoat®) in clinically relevant *in vivo* chronic burn wound model. Importantly, it has shown that no one product has superior efficacy. Rather, varying mechanisms of actions in each of the treatments resulted in a common better healing trajectory than if the wound were untreated. This concept supports the complexities of wound healing pathways and more relevantly the multiple aetiologies involved in chronic wounds.

A few salient points from this study, technical and methodological flaws and foreseeable future studies warrant discussion. This discussion will concentrate on the following issues:

- ❖ Relevance of the model to the study and use of outcome variables – epithelial gap, cellular proliferation and semi-quantitative cultures
- ❖ What answers do we have from the study
- ❖ What were the study flaws
- ❖ What future studies are needed to build on answers gained in this work

Epithelial gap closure as a model

This study demonstrated the closure of a wound by means of epithelial migration. Epithelial gap measurements showed that NCS (Acticoat®) had a significantly greater epithelial closure at Day 21 ($p < 0.05$) compared to the control group. The measurement of epithelial closure over a raw surface is ultimately the end point of wound healing. Proteomics and genomics allow a far more sensitive and in depth look at the events around epithelialisation. The requisite technical expertise and prohibitive costs did not allow us to use these methods. The excisional burn wound model was modified from existing models. Galiano *et al.*[41] had demonstrated that epithelialisation could be the major process in wound closure in a murine model, using a splint. This study used a Sprague-Dawley rat model with splinting, to negate wound contraction. The rat model allowed an easier, cost effective model in our scenario for the number of animals that were used (128), especially considering the time period of 21 days. The pitfalls of this method are noted in Coulombe's explanation on epithelialisation in a wound model.[44] He noted the histological sectioning effects (shift and tear) on symmetry of a wound and the lack of an ideal animal model were factors that confounded the study of the epithelial advancing edge. An *ex vivo* skin explant technique or the use of skin substitutes has been studied as an acceptable comparison.[45] The prohibitive costs of this method did not

allow for this methodology in this study and a simpler histological alternative (epithelial gap) was chosen instead.

Anti-inflammatory effects of BF and NCS

Cellular proliferation measured by Ki-67 staining revealed that both BF (Flavonix®) and NCS (Acticoat®) showed varying degrees of anti-inflammatory activity with proliferation in the basal epidermal layer (stratum basale) being suppressed. BF (Flavonix®) showed a significant suppression of proliferation at Day 21 in certain HPF's ($p < 0.05$). A burn wound was created prior to it being excised four days later. Burn wounds induce inflammatory changes, with a central zone of necrosis surrounded by concentric adjacent zones of stasis and hyperaemia in a three dimensional spatial arrangement in tissue.[46] The excision of the necrotic area still meant that the surrounding tissue had sufficient inflammation and that the treatments used would be able to exert a significant effect on the host inflammatory response. The dampening of the inflammatory response by both treatments was associated with a decrease in epithelial gap distance, suggesting that the anti-inflammatory effect of these products may be beneficial to a healing wound. In a chronic wound scenario, where exaggerated inflammation is perpetuating a cycle of ECM breakdown, this is a positive finding. Wright *et al.* have substantiated the association between anti-inflammatory effects of these treatments (decreased MMP levels in NCS treated wounds) and their direct relationship to improving early wound healing events.[24] Falanga *et al.*[12] showed that cellular migration was a consequence of a healthy granulation bed as well, therefore one can deduce that the anti-inflammatory effects likely result in a change in the ECM of this granulation tissue. Molecular technique (gene targeting and gene silencing *RNAi*[47]) confirmation of this activity would reveal the changes in inflammatory markers better defining the processes which occur at the

epithelial advancing front, distinguishing cell proliferation, migration and differentiation and the signals which control these processes.

Bacterial burden and its role in healing

The bacterial burden measured in this study showed that bacteria were not eradicated by the treatments. Only BF (Flavonix®) showed a significant effect on *P. aeruginosa* ($p = 0.038$) at Day 21 compared to the control group. The number of *S. aureus* colonies remained virtually the same with the NCS (Acticoat®) group showing a slightly higher percentage of wounds over the threshold limit. Yet the healing trajectories of the wounds in both treatment groups showed improved healing with respect to early events.

NCS (Acticoat®) works more effectively in combination with other agents such as lactoferrin and xylitol.[23] NCS (Acticoat®) on its own does not have sufficient anti-biofilm activity.[48] Flavonix® would theoretically work better in combination with other anti-microbial agents. The anti-biofilm property would allow secondary agents to take effect against bacteria in planktonic rather than sessile states. In this study four animals were treated with a combination of Acticoat® and Flavonix® to POD 14. These findings showed a reduction in *P. aeruginosa* in two of the four specimens to below threshold and more importantly a reduction in *S. aureus* in all of the specimens to below threshold levels. This data cannot be validated due to an insufficient sample size. Further studies are warranted in assessing the outcomes of the two treatments being used simultaneously.

S. aureus is found in far greater numbers and *P. aeruginosa* has the ability to secrete proteins that readily allow it to protect itself in a biofilm state.[49] An important observation in this study was the colonization and infection of non-inoculated animals with equal concentrations of bacterial burden as early as Day 10. This finding highlights the importance of a clinically relevant model. Our model aimed to house and care for the wounds in a way that would be comparable to any chronic wound treated in an outpatient setting. The use of *P. aeruginosa* and *S. aureus* mimicked commonly found organisms in clinical cases of chronic wounds.[49]

Bacterial load or bioburden is known to cause an increase in proteolytic activity and a greater inflammatory response with increase neutrophils in the wound area. This response contributes to the pathophysiology of a chronic wound and the eradication or disruption of biofilms has been shown to improve healing[50]. Biofilms are commonly found in chronic wounds (60% – 80%)[13]. No one particular method has been standardized for the purpose of monitoring biofilm formation and degradation. Our study methods used semi-quantitative cultures as a crude indirect method of assessing the bacterial bioburden. Although not directly indicative of biofilm the analysis does quantify bioburden in a manner that is clinically relevant. This method was chosen as it has a 100% positive predictive value and 93,7% negative predictive value of clinical sepsis in a wound and simultaneously avoids the intensive and expensive exercise of counting exact colonies.[19] The assessment of bacterial load does not necessarily translate to a direct assessment of the biofilm. Further work is required in assessing a more accurately quantifiable target within biofilms in order to evaluate the effects of these dressings on biofilm.

Potential improvements to this study and future work

Wound healing events in an in-vivo chronic wound model have been described in this study. The effect of these treatments has been shown to be equivalent when compared across variables, with no one treatment showing superiority. Wound healing outcomes require more sensitive detection techniques. PCR and gene amplification provides a means to better define inflammatory processes by measuring specific markers (IL's, MMP's etc.). Secondly, gene marker identification of the products found within biofilms and those bacteria responsible for the biofilm structures will allow a new dimension in evaluating wounds. Instead of assessing common end pathways, the possibility exists to assess and modulate causative factors at the problematic site. This data can then be compared to macroscopic outcomes such as epithelial gap reduction to give a more significant and reliable indication of treatments and their effects.

Conclusion

Taken as a whole, the results indicate that control of inflammation in wound healing is as important as addressing its bacterial cause. The initiated inflammatory response to bacterial burden needs to be balanced. The inflammation is key to the healing cascade. When this response is exaggerated it is self-destructive. The eradication of all bacterial burden is not necessary to achieving wound healing. Both BF (Flavonix®) and NCS (Acticoat®) have shown a modulation of this response, without the concomitant absence of bacteria. Additional studies are required to better define this modulation as well as the impact of NCS and BF on biofilms.

Appendix A

Results of Porcine Deep Partial Thickness Wound Model, of 3 treatment groups; Flavonix® (Medika, SA), Intrasite® (Smith and Nephew, UK) and a negative control group

Ethics clearance number : 2008/23/05

Aim :
To demonstrate that Flavonix® gel does not inhibit epithelialisation

Method :
A deep partial thickness wound model was used in 3 female large white pigs. Wounds were treated with Flavonix® gel, Intrasite® gel and a negative control group. Punch biopsies were taken at days 4, 7 and 10 from within the wounds and haemotoxylin and eosinophil (H&E) sections prepared. Epidermal thickness measurements were taken along 11 equidistant points in the completely healed wounds and an average epidermal thickness measured.

Results :

At day 10 there is a significant difference in epidermal thickness between the Flavonix® group and the other Intrasite® ($p = 0.000033$) as well as between the Flavonix® and control group ($p = 0.01$).

Conclusion :
Flavonix® gel showed no adverse effects on epidermal thickness in a deep partial thickness porcine wound model at Day 10.

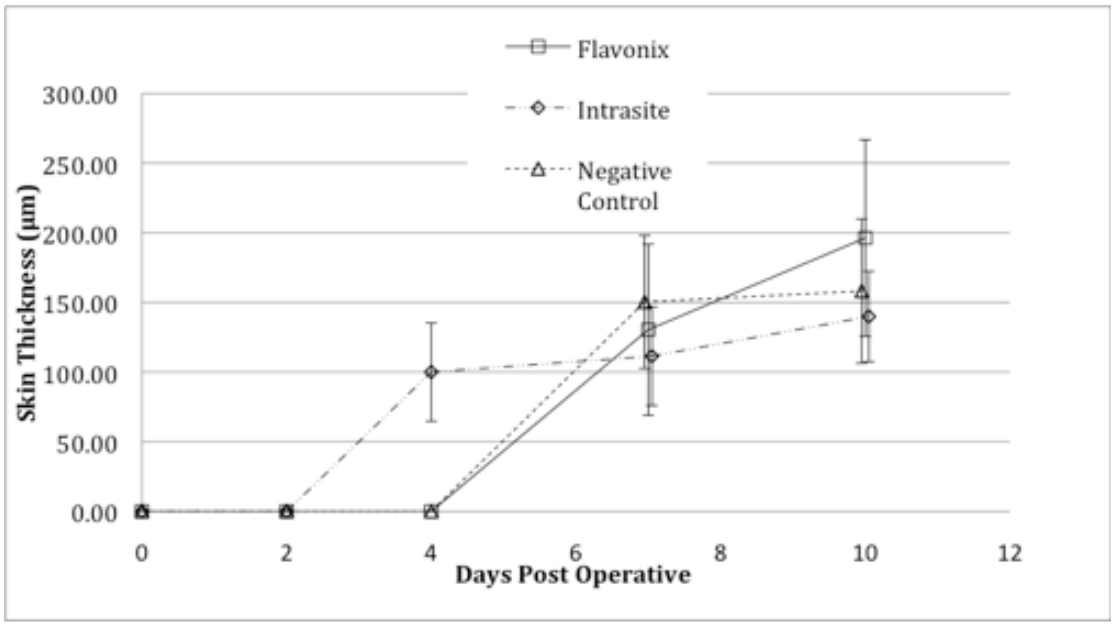


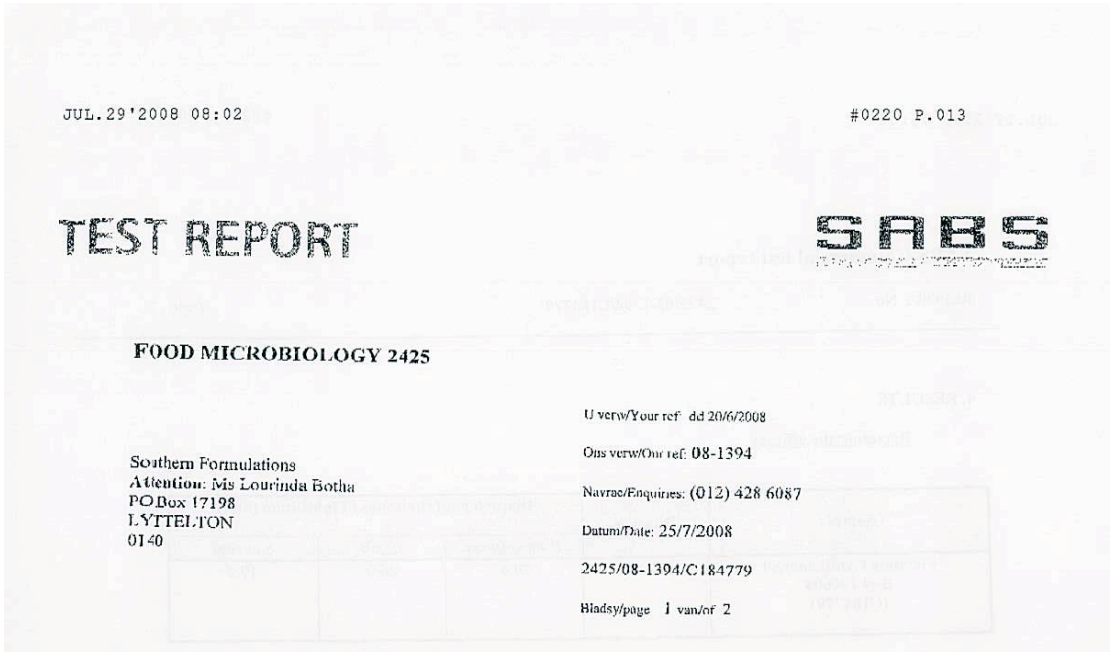
Figure : Epidermal thickness of deep partial thickness wounds over 10 days. Flavonix® gel shows increased epidermal thickness to control groups at Day 10.

Appendix B

Table : Ingredients of the Bolton Broth (Oxoid, SA)

Bolton Broth Ingredients : (Oxoid, South Africa)	
Ingredient	**Typical formula (g/L)**
Meat Peptone	10.0
Lactalbumin hydrolysate	5.0
Yeast Extract	5.0
Sodium Pyruvate	0.5
Alpha-Ketoglutaric Acid	1.0
Sodium metabisulphite	0.5
Sodium carbonate	0.6
Haemin	0.01

Appendix C



JUL.29'2008 08:02 #0220 P.013

TEST REPORT

SABS

FOOD MICROBIOLOGY 2425

Southern Formulations
Attention: Ms Lourinda Botha
PO Box 17198
LYTTELTON
0140

U verw/Your ref: dd 20/6/2008

Ons verw/Our ref: 08-1394

Navrae/Enquiries: (012) 428 6087

Datum/Date: 25/7/2008

2425/08-1394/C184779

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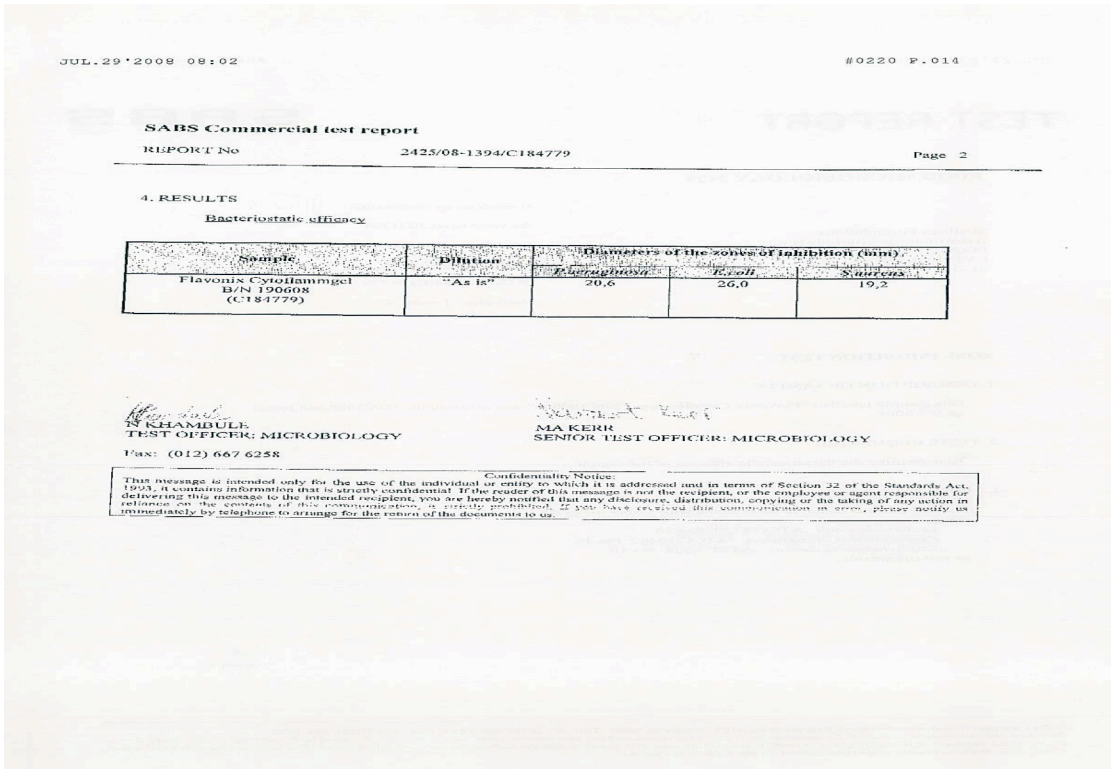

JUL.29'2008 08:02 #0220 P.014

SABS Commercial test report

REPORT No 2425/08-1394/C184779 Page 2

4. RESULTS

Bacteriostatic efficacy

Sample	Dilution	Diameters of the zones of inhibition (mm)		
		Staphylococcus	*Bacillus*	*Shigella*
Flavonix Cytoflammgel B/N 190608 (C184779)	"As is"	20,6	26,0	19,2

N KHAMBULE
TEST OFFICER: MICROBIOLOGY

MA KERR
SENIOR TEST OFFICER: MICROBIOLOGY

Fax: (012) 667 6258

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