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Development of a silk protein based wound dressing and its effect on wound healing in rats

by

Jeané Olivier

Thesis submitted in partial fulfillment of the requirement for the degree of

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Supervisor: Prof. E. Pretorius

Department: Anatomy

Degree: MSc (Anatomy with specialization in Cell Biology)

ABSTRACT

Silk has been used in textile production for centuries and in medicine, as sutures for decades. Sericin and fibroin are the two primary silk proteins. Silk has been shown to improve wound healing. Copper ions have also been documented to have healing properties. The novel silk protein based wound dressing donated by Southern Formulations (Pty) Ltd. for evaluation contains sericin, fibroin and copper ions. The aim of this study was to investigate the effect of silk proteins and copper ions combined in the form of a novel silk protein based wound dressing on burn wound healing. In the current thesis the effect of this novel silk protein based wound dressing on wound healing was investigated first by evaluating whether the dressing was cytotoxic. After determining its cytotoxicity a Wistar rat burn wound was established and utilized to investigate the effect of the novel silk protein based wound dressing on wound healing. Its effect was evaluated at the site of the wound as well as on a systemic level.

The novel silk protein based wound dressing's cytotoxic effect was evaluated on a human fibroblast primary cell culture by exposing the cells to serial dilutions of

an extract prepared from the dressing. The results indicated that the novel silk protein based wound dressing is not cytotoxic up to a concentration of 25 mg/µl. No IC_{50} value could be found. The Wistar rat burn wound model was established with Butorphanol as part of the analgesic regimen. Histological investigation, by means of H & E and Masson's Trichrome staining, showed that the novel silk protein based wound dressing improves epithelialization and neovascularization. On a systemic level the novel silk protein based wound dressing decreased the inflammatory process significantly. This was determined by white blood cell counts of blood smears prepared from the blood samples taken from the treatment group. The white blood cell count of the treatment group was compared with that of the control group. The white blood cell counts also showed that the dressing did not initiate an allergic response. It was also determined whether the novel silk protein based wound dressing has an impact on fibrin network architecture in circulating plasma, and whether a local burn injury treated with the novel wound dressing will change clot ultrastructure. This was evaluated by scanning electron microscopy of fibrin networks created from the rat platelet rich plasma prepared from the treatment group and compared with that of the control group. The results indicated that the novel silk protein based wound dressing did not affect clot ultrastructure.

It is therefore concluded that the novel silk protein based wound dressing, containing copper ions and silk proteins sericin and fibroin, improves the formation of granulation tissue by improving epithelialization and neovascularization. It is also concluded that inflammation is statistically significantly decreased in the group of rats treated with the novel silk protein based wound dressing. Additionally, the novel silk protein based dressing does not cause an allergic reaction after primary exposure and does not change fibrin network formation or clot ultrastructure.

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DECLARATION

I, Jeané Olivier, hereby declare that this research dissertation is my own work and has not been presented by me for any degree at this or any other University.

Signed: ……………………………..

Date: ……………………………..

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List of Abbreviations, Symbols and Formulae

%	Percentage
°C	Degrees Celsius
4-HNE	4-hydroxynonenal
ATIII	Antithrombin III
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
cm	Centimeter
cm^2	Square centimeter
CO2	Carbon dioxide
COX-2	Cyclooxygenase-2
DMBA	7,12-dimethylbenzanthracene
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
FGF-1	Fibroblast growth factor-1
g	Gram
H & E	Hematoxylin and Eosin
h	Hour
H_2O	Water
HeLa	Human cervical adenocarsinoma cells
HMDS	Hexamethyldisilazane
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-β1	Interleukin-β1
IM	Intramuscular
kDa	Kilodalton
mg	Milligram
mg/kg	Milligram per kilogram
mg/ml	Milligram per milliliter
mg/µl	Milligram per microliter

min	Minute(s)
mJ/cm^2	Millijoule per square centimeter
ml	Milliliter
mm	Millimeter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide
nm	Nanometer
nmol	Nanomole
PAA	Poly(acrylic)acid
PAI	Plasminogen activated inhibitor
PBS	Phosphate buffered saline
PC12	Pheochromocytoma clonal cell line
PCF	PVA/chitosan/fibroin
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PRP	Platelet rich plasma
PVA	Polyvinyl alcohol
red-ox	Reduction-oxidation
rpm	Revolutions per minute
RuO_4	Ruthenium tetraoxide
SD	Standard deviation
sec	Second(s)
SEM	Scanning electron microscopy
Sericin-S	Small sericin
Sericin-L	Large sericin
TAFI	Thrombin activatable fibrinolytic inhibitor
TFPI	Tissue factor pathway inhibitor
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
U/ml	Units per millilitre
UPAUCC	University of Pretoria's Animal Use and Care Committee
UPBRC	University of Pretoria Biomedical Research Centre

UV	Ultraviolet
UVB	Ultraviolet B light
VEGF	Vascular endothelial growth factor
µg/cm^2	Micrograms per square centimeter
µl	Microliter
µM	Micro molar
µm	Micrometer
x	Times

Chapter 1: Introduction

The novel silk protein based wound dressing donated by Southern Formulations (Pty) Ltd. for evaluation contains three actives. The primary actives are the proteins, sericin and fibroin and the secondary active is copper ions. Sericin and fibroin are the two main components of silk from the domesticated silk worm, *Bombyx mori*. Silk is biomedically defined as a natural polymer. Fibroin forms the inner core protein and the outer coating of the silk fiber is formed by sericin (Zhang, 2002). Both silk proteins and copper have been documented to have properties that would improve wound healing.

Silk has been used in textile production for centuries. *Bombyx mori* silk fibers have been the primary silk material used as a biomaterial, mainly in the form of sutures. For decades silk has been used clinically and proven to be effective. Only recently have silk proteins been used for a range of other biomedical applications. Presently silk has applications from clinical repairs to being used *in vitro* as scaffolds for tissue engineering (Altman *et al*., 2003).

Literature reports on silk proteins being used for enzyme immobilization (Miyairi and Sigiura, 1978), contact lenses, artificial blood vessels (Zhang, 2002), mucoadhesive drug delivery systems (Ahn *et al*., 2001), medical materials and wound dressings (Yoshii *et al*., 2000). Silk proteins have also proven to be effective and valuable in cell culture.

Sericin and sericin hydrosylates can protect cells and proteins from freezing stresses and can therefore be used as a cryoprotectant (Kazuhisa *et al*., 2001). It has also been documented that sericin can be used as a substitute or alternative supplement for serum in certain cell cultures (Ogawa *et al*., 2004; Terada *et al*., 2005). It has also been reported that sericin enhances attachment of cultured human skin fibroblasts (Tsubouchi *et al*., 2005) and accelerates cell proliferation

(Terada *et al*., 2002). In cell biology fibroin has mainly been utilized as a scaffold or matrices in tissue engineering due to its unique properties (Gil *et al*., 2007). Fibroin has been used as a cell support system for various different cell types from osteoblasts (Lu *et al*., 2007; Pra *et al*., 2005) to astrocytes (Pra *et al*., 2005).

Not only have these silk proteins proven their worth in cell culture, but sericin and fibroin have also shown good results in studies evaluating their ability to improve wound healing. Sericin has been reported to improved epithelialization and collagen deposition while also decreasing inflammation (Aramwit and Sangcakul, 2007). Although fibroin is mostly utilized for its good structural properties, it has also been shown to have wound healing properties. It has been reported that fibroin decreases healing time when applied topically. Additionally fibroin has been documented to also improve collagenization (Sugihara *et al*., 2000). Literature therefore suggests that silk proteins may be beneficial for treating wounds such as those formed by thermal injury (Aramwit and Sangcakul, 2007; Sugihara *et al*., 2000).

It is known that thermal injury has a systemic effect giving rise to certain complications (Kowal-Vern *et al*., 2000). It has been documented that burn injury induces the dermal production of pro-inflammatory mediators, resulting in ongoing wound inflammation and tissue edema (Ipaktchi *et al*., 2006). It is also known that injury due to burn has an impact on coagulation and haemostasis (Lavrentieva *et al*., 2008). Furthermore it has been documented that the silk fibroin protein is able to bind to components of the clotting cascade such as fibrin and fibrinogen. Therefore silk has been found highly thrombic. However, in most cases, this response is moderate and subsides with time (Altman *et al*., 2003). Additionally, silk, like most proteins, is a potential allergen, causing a type I allergic response in some cases. This indicates that silk may have an effect on the immune system (Kurosaki *et al*., 1999; Altman *et al*., 2003). For this reason, it may be reasoned that silk proteins may have a systemic effect when applied locally.

The predominant natural polymer used to treat wounds at the wound site, such as burns, is collagen or collagen products (Lu *et al.*, 2007). These products support wound healing by laying down a matrix that forms a favourable environment for the deposition of new tissue. It is also thought to attract cell types necessary for healing. These products are available in particle and sheet form. The collagen used are from human, porcine, and bovine sources. Most of these collagen products work by absorbing wound fluid and as a result forms a soft biodegradable gel over the wound surface that also helps to maintain wound moisture (Fonder *et al.*, 2008). However, the range of physical properties of collagen is limited (Lu *et al.*, 2007). Another drawback of collagen is the regulatory aspects and cost (Eming *et al.*, 2002). However, it has already been shown *in vitro* that silk proteins can successfully be used as a substitute for collagen (Minoura *et al.*, 1995; Zhang, 2002; Tsukada *et al.*, 1999). Even though silk proteins are a sufficient substitute, collagen is still more effective. Therefore an additive is necessary (Zhang, 2002; Tsukada *et al.*, 1999).

As mentioned previously the novel silk protein based wound dressing also contains copper ions. Copper is an important trace element with numerous physiological functions. This is also true in the process of normal wound healing. Endothelial cell migration and proliferation is an important part of the wound healing process (Cohen *et al.*, 1992; Fonder *et al.*, 2008). Copper is necessary for endothelial cell activation as well as proliferation and migration. Copper has been documented to improve wound healing by activating angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (Nasulewicz *et al.*, 2004). Copper is also important for fibronectin stabilization (Borkow *et al.*, 2008) and cross-linking elastin and collagen (Gupte and Mumper, 2009). Therefore, according to the literature, it is clear that both silk proteins and copper ions have a positive effect on wound healing. However, no literature could be found on the effect of a combination of these actives.

From this the question arises whether the novel silk protein based wound dressing, that contains a combination of silk proteins and copper ions, could improve the treatment of a partial thickness burn wound. The overall aim of the study was to investigate the effect the novel silk protein based wound dressing on epithelialization and collagen deposition, as well as the systemic effect on white blood cell counts and fibrin network architecture using a Wistar rat burn wound model.

Therefore, the following research objectives will be investigated:

- The establishment of a burn wound protocol using Wistar rats.
- To determine the cytotoxic effects of the novel dressing.
- To utilize the Wistar rat burn wound model to investigate the effect of the novel dressing on granulation and epithelailization.
- To utilize the Wistar rat burn wound model to investigate the effect of the novel dressing on collagen deposition.
- To determine the systemic effect on white blood cell counts after treatment with the novel dressing.
- To determine whether the novel dressing has an impact on fibrin network architecture.

The mentioned research objectives where used to determine the cytotoxicity of the novel silk protein based wound dressing preceding the animal study. The established animal model was then used to evaluate the effect of the novel silk protein based wound dressing on the healing of a burn wound, on both a macroscopic and microscopic level, by comparing a treatment group to a control group. Similarly, the established animal model was used to investigate the systemic effects of a burn wound treated with the novel silk protein based wound dressing through evaluating the white blood cell counts and fibrin network architecture.

Chapter 2: Literature Review

2.1 INTRODUCTION:

In the current thesis the effect of a novel silk protein based wound dressing on burn wound healing will be investigated using a Wistar rat burn wound model. In this literature review the different components of the dressing as well as their individual effect on wound healing will be discussed. Finally the role of animal models in the study of burn wound healing will be discussed as well as the reasons for selecting the current animal model.

2.1.1 The novel silk protein based wound dressing:

Silk from the domesticated silk worm, *Bombyx mori*, is a natural polymer that is mainly composed of two proteins. An inner core protein known as fibroin and another protein that forms the outer coating of the silk fiber known as sericin. The primary actives of the novel silk protein based wound dressing studied in this thesis are fibroin and sericin. The dressing also contains trace elements of copper ions as a secondary active.

Sericin encloses the fibroin fiber with successive sticky layers that help in the formation of a cocoon (Zhang, 2002). In order to perform this function sericin has adhesive and gelatin like properties**.** Sericin is a macromolecular protein with a molecular weight ranging from about 10 kDa to over 300 kDa. The sericin protein is made up of 18 amino acids most of which have strongly polar side groups such as hydroxyl, carboxyl, and amino groups. In addition, the amino acids serine and aspartic acid constitute approximately 33.4% and 16.7% of sericin, respectively.

Serine is the main amino acid of the natural moisture factor in human skin. As mentioned above sericin consists of more than 30% serine and therefore sericin is an exceptional moisturizing agent (Aramwit and Sangcakul, 2007). Additionally,

other properties of sericin includes its antibacterial, antioxidant and anticancer activity, it also protects against ultra violet (UV) light (Zhaorigetu *et al*., 2003; Aramwit and Sangcakul, 2007).

Sericin has other characteristic properties as well. Sericin enhances the attachment of primary cultured human skin fibroblasts (Tsubouchi *et al*., 2005)**,** it has anti-inflammatory properties (Aramwit and Sangcakul, 2007), sericin can be used as a serum replacement for the culture of islet cells (Ogawa *et al*., 2004) and also to improve serum-free mammalian cell culture (Terada *et al*., 2005) and sericin can also improve cell survival during the cryopreservation when added to the medium (Terada *et al*., 2003).

Sericin is a water soluble macromolecular protein. When it is dissolved in a polar solvent the size of the resulting sericin molecules depend on the pH, temperature and processing time (Aramwit and Sangcakul, 2007). The lower molecular weight molecules, either sericin peptides or sericin hydrosylates, are normally used for cosmetic purposes, such as hair care products and other health products. They are soluble in cold water and can be recovered during the early stages of raw silk production. The higher molecular weight sericin molecules are soluble in hot water and can be recovered during the later stages of raw silk production known as degumming of the silk. The higher molecular weight sericin molecules are most often used in medical biomaterials such as degradable biomaterials, biomembranes and hydrogels (Zhang, 2002).

The other major protein found in silk is known as fibroin. Fibroin is the structural protein of silk fibers. It consists of heavy and light chain polypeptides of 350 kDa and 25 kDa respectively and is connected by a disulfide bond. Fibroin is mainly made up of the amino acids glycine, alanine and serine. The high-molar-mass chains of fibroin contain Gly–Ala–Gly–Ala–Ser residues that are capable of organizing into crystalline β-sheet structures. This arrangement creates the stability and mechanical features of the fibers (Kim et al., 2005). Once the sericin coating in

raw silk is removed, fibroin is soluble in neutral salt solutions such as lithium bromide and calcium nitrate. The dissolved fibroin can then be regenerated in film, fiber, and scaffold form (Gil *et al*., 2007).

Regenerated fibroin is fully amorphous, with its chains adopting a random-coil conformation, while naturally occurring silk fibroin's structure is in a β-sheet conformation. Dehydration of the regenerated fibroin in an alcohol promotes solvent-induced fibroin crystallization. The silk fibroin chains transform back from random-coil to β-sheet conformation.

According to the literature regenerated silk fibroin has been successfully blended with other macromolecular reagents, such as chitosan, cellulose (Sashina *et al*., 2007), polyvinyl alcohol (Wang *et al*., 1998; Yoshii *et al*., 2000), polyacrylic acid (Ahn *et al*. 2001) and nylon-6, 6 (Gil *et al*., 2007). Blending fibroin with other polymers enhances its properties allowing even more applications.

Fibroin has potential as a biomaterial or scaffold in tissue engineering because of its remarkable mechanical properties, biocompatibility and biodegradability (Kim et al., 2005). These unique physiochemical characteristics have facilitated its many uses, including uses such as an oral dosage gel form (Ahn *et al*., 2001; Park *et al*. 2002), enzyme–immobilizing membranes (Miyairi and Sigiura, 1978; Asakura *et al*., 1992; Park *et al*. 2002) and as cell culture matrices (Park *et al*. 2002; Minoura *et al*. 1995; Tsukada *et al*., 1999; Kim et al., 2005; Fan et al., 2008). The novel silk protein based wound dressing supplied by Southern Formulations (Pty) Ltd. for evaluation is a regenerated film dressing that acts as an actives delivery system.

2.1.2 The history and applications of silk as a biomaterial:

Silk has been used in textile production for centuries. Silks are generally defined as protein polymers. The most comprehensively characterized silk is the silk from the domesticated silk worm *Bombyx mori.* *Bombyx mori* silk fibers have been the primary silk material used as a biomaterial, mainly as sutures. For decades silk has

been used clinically and proven to be effective. Only recently have silk proteins been applied biomedically as matrices. These matrices are useful for a range of applications from applications in clinical repairs to being used *in vitro* as scaffolds for tissue engineering (Altman et al. 2003).

At this stage silk-based biomaterials have been demonstrated to generate functional tissue replacements for a range of mesenchymal tissues. These tissues include bone, cartilage, ligaments and adipose tissue. In these applications silk has proven to have low immunogenicity, slow degradation rates, good mechanical properties and no bioburdens (Mauney *et al.*, 2007).

In 1978 Miyairi and Sigiura made a cross-linked sericin film that could be used for enzyme immobilization. In 1988 Hirotsu and Nakajima used both sericin and fibroin to make a membrane for use in separation processes. They prepared an insolubilized silk membrane that was used to separate water from a mixture of water and alcohol. It was later found that siricin and fibroin membranes have excellent oxygen permeability and functional properties that is comparable with human cornea (Murase, 1994). Therefore silk membranes proved to be useful as contact lenses, highly elastic artificial blood vessels and other prostheses (Zhang, 2002).

Silk protein based membranes have also proved to be effective in cell culture. In 1995 Minoura and co-workers prepared a membrane composed of sericin and fibroin as an effective substrate for the proliferation of adherent animal cells. It can therefore be used as a substitute for collagen (Minoura *et al.* 1995; Zhang, 2002). Their research is supported by the findings of Tsukada and co-workers in 1999.

On a biochemical level, by sulfonation treatment of sericin and fibroin, silk proteins can be made into a biomaterial with anticoagulant properties. In 1997 Tamada stated that sulfonated silk can potentially be used as a substitute for heparin to treat surfaces of medical devices. In 1998 Kato and co-workers also found that silk sericin suppresses lipid peroxidation and inhibits tyrosinase activity *in vitro*.

However, there are few documented reports on the biological functional properties of silk proteins at a molecular level (Zhang, 2002).

Also in 1998, Wang and co-workers investigated the structural and physical properties of a polyvinyl alcohol (PVA) and sericin blended hydrogel membrane. In 2000 Yoshii and co-workers reported that sericin or fibroin combined with PVA hydrogels have excellent moisture absorbing and elastic properties. They therefore concluded that such a hydrogel can be used in medical materials and wound dressings (Yoshii *et al.*, 2000; Zhang, 2002). Around the same time, in 1999 Tsubouchi and co-workers developed an occlusive sericin-fibroin based wound dressing that was used to accelerate healing. Additionally their wound dressing could be peeled off easily without causing damage to the underlying newly formed tissue.

Silk protein membranes can also be used as a mucoadhesive polymer. In 2001 Ahn and co-workers composed a poly(acrylic) acid (PAA) and sericin blended polymer that could potentially be used as a mucoadhesive drug delivery system. Also in 2001 Kazuhisa and co-workers found that sericin and sericin hydrosylates can protect cells and proteins from freezing stresses and can therefore be used as a cryoprotectant. Silk proteins have been used for other applications in cell culture as well.

In 2004, in the field of cell biology, Ogawa and co-workers improved the culture procedure of islet cells by substituting serum with sericin as an alternative supplement**.** In 2005 Tsubouchi and co-workers reported that sericin enhances attachment of cultured human skin fibroblasts. In the same year Terada and co-workers (2005) reported that sericin improves serum free mammalian cell culture.

In 2004 there were also advances in the field of tissue engineering. Pra and co-workers (2004) reported that silk fibroin nonwovens effectively guided reticular connective tissue engineering for regeneration or repair applications. In 2005 Kim

and co-workers prepared a porous aqueous derived silk fibroin scaffold without using organic solvents or chemical cross-linking that would potentially have better biocompatibility. Then, in 2006, Wang and co-workers successfully used silk scaffolds for *in vitro* cartilage tissue engineering using human articular chondrocytes.

In 2007 the use of silk proteins for tissue engineering improved even more. Mauney and co-workers (2007) used a silk fibroin three dimensional scaffold to engineer adipose-like tissue *in vitro* and *in vivo* making use of human bone marrow and adipose derived mesenchymal stem cells. They found that three dimensional silk fibroin scaffolds provide long term structural integrity to promote the maintenance of soft tissue *in vivo*. Lu and co-workers (2007) evaluated the cytocompatibility and blood compatibility of multifunctional fibroin-collagen-heparin scaffolds. They concluded that these hybrid scaffolds were indeed cytocompatible and blood compatible with outstanding porous structure and mechanical properties. These scaffolds hold promise for blood contact tissue engineering (Lu *et al*., 2007). Lovett and co-workers (2007) developed silk fibroin microtubes for blood vessel engineering and repair. Yang and co-workers (2007a) developed and evaluated a silk fibroin based nerve grafts for peripheral nerve regeneration. Then, in 2008, Fan and co-workers used a gelatin/silk fibroin hybrid scaffolds to successfully co-culture fibroblasts and mesenchymal stem cells. They concluded from their findings that it is feasible to use this system in ligament tissue engineering. In the same year Wang and co-workers (2008) also reported that it is feasible to utilize drug loaded silk fibroin for vascular stent coatings.

Also in 2007, Aramwit and Sangcakul evaluated the effect of sericin cream on wound healing utilizing a rat animal model. They concluded that sericin has wound healing properties without initiating an allergic reaction. Additionally they found that the treatment group showed less inflammation when compared to the control.

2.2 APPLICATIONS OF SILK PROTEINS IN CELL CULTURE:

In the past tissue engineered matrices were defined as a material that only functioned as an inert structural support for cell attachment. However, recent tissue engineered matrices are more complex, serving as a dynamic environment for tissue development. Contemporary tissue engineered matrices should have excellent biocompatibility, high porous structure, suitable mechanical properties and reactive areas for cell adhesion and growth. Another characteristic that is beneficial of today's matrices is that they provide an inductive environment that can release bioactive factors. In order to accomplish this, natural polymers create a more responsive environment by allowing degradation and remodeling by cell-secreted enzymes and have thus proven to be superior to synthetic polymers (Lu *et al*., 2007).

Collagen is the best known natural polymer and has been used comprehensively in the past. Even though collagen has proven to be effective, its range of physical properties is limited (Lu *et al*., 2007). Recently silk fibroin has also proven to be an effective biomaterial for tissue engineered matrices. Fibroin is also able to form porous, biocompatible and mechanically robust scaffolds (Gil *et al*., 2007). Fibroin has been used as a cell support matrice for osteoblasts (Lu *et al*., 2007; Pra *et al*., 2007), chondrocytes (Wang *et al*., 2006), adipose cells (Mauney *et al*., 2007), hepatocytes, fibroblasts (Lu *et al*., 2007; (Pra *et al*., 2007), keratinocytes, endothelial cells, and astrocytes (Pra *et al*., 2007).

Fibroin has also been used as a scaffold for ligament tissue engineering due to its impressive biocompatibility, biodegradability, minimal inflammatory reactions and mechanical properties (Lu *et al*., 2007). Due to its ability to promote cell adhesion and growth, fibroin has been studied extensively as a potential biomaterial forming either the core or by coating the surfaces of scaffolds aimed at tissue engineering, regeneration and repair (Pra *et al*., 2007).

Unger *et al.* evaluated the ability of a biomaterial consisting of a non-woven fibroin net, to support the growth of human cells. They evaluated the adherence and growth of human cells from numerous tissues including endothelial, epithelial, fibroblast, glial, keratinocyte and osteoblast cells. The cells were stained with calcein-AMand and then examined by confocal laser microscopy. Adherence and growth were also evaluated by electron microscopy. They found that all the cells readily adhered and spread over the individual fibers of the nets, and most cells grew and survived on the nets for at least 7 weeks. The cells not only grew and covered the individual fibers of the net but also bridged the gaps between individual fibers forming tissue-like structures. After observing the cells with scanning electron microscopy (SEM), a tight association of individual cells with the fibers of the nets was seen. After removing the cells from the net no evidence could be found that the growth of cells changed the structure of the fibers. They concluded that silk fibroin nets are highly human cell-compatible. They also stated that these silk fibroin nets could be a useful new scaffolding biomaterial applicable for a wide range of target tissues in addition to supporting endothelial cells required for the vascularization of the newly formed tissue (Unger *et al*., 2004). Fibroin is functional in cell culture due to its mechanical properties and can therefore be used as cell culture matrices. Sericin, on the other hand, have shown to have other properties useful in cell culture.

Terada *et al.* evaluated small sericin, which they called sericin-S, on cell proliferation in 2002. Sericin-S has a molecular weight ranging from 5 kDa to 100 kDa. They reported that sericin-S is a cell culture supplement used to accelerate cell proliferation (Terada *et al.* 2002). In 2005 they evaluated sericin large or sericin-L, prepared under non-hydrolyzing conditions and ranging from 50 to 200 kDa. They reported that sericin-L also accelerated cell proliferation, but its effects were less when compared to those of sericin-S. Additionally the reported that sericin-S successfully accelerated the proliferation of hybridoma cells in various serum free media. This finding implied that the mitogenic effect of sericin is independent from media. Also, sericin-S successfully stimulated the proliferation of an established T

lymphocyte cell line, under interleukin-2 (IL-2) starvation conditions. Therefore they concluded from their results that sericin, especially sericin-S, improves serum-free mammalian cell culture. Terada *et al.* revealed that sericin is able to enhance cell proliferation (Terada *et al*., 2005), while Tsubouchi and co-workers (2005) reported that sericin enhances cellular adhesion.

Tsubouchi and co-workers (2005) evaluated the effect of sericin on cultured human skin fibroblasts. The cells were culture in Petri dishes coated with 2.5 $\mu g/cm^2$ of sericin. After 72 hours the cell count was compared with a non-sericin control. After 72 hours the cell count of the sericin treated cells was 250% more than the non-sericin control. However, the proliferation rates were the same when comparing the two. Therefore, Tsubouchi and co-workers (2005) concluded that the increase in cell count was due to enhanced initial attachment. Additionally, phase contrast microscopy observations revealed that cells grown on the sericin coated Petri dishes had a well extended fibrous shape while the cells of the control revealed a round shape. They finally concluded that sericin has potential as a scaffold or matrix to culture human fibroblasts.

Literature suggests that the silk proteins sericin and fibroin have promising applications in cell culture. However, studies have also been done to evaluate the effect of these proteins on wound healing.

2.3 SILK PROTEINS FACILITATE WOUND HEALING:

In 2003 Zhaorigetu and co-workers conducted a study to assess the protective or antioxidant effect of sericin on ultraviolet B light (UVB) induced acute damage and tumor promotion in mouse skin. They did two experiments. In the first experiment they evaluated the photoprotective effect of sericin. They made use of hairless mice and treated them with 180 mJ/cm^2 of ultraviolet B light once daily for 7 days. The treatment for 7 days caused red sunburn lesions of the skin. One group of ten mice was treated with a dose of 5 mg of sericin after the UVB

treatment, while the control group received bovine serum albumin (BSA). This protein was chosen because *in vitro* studies done in the past have shown sericin to have a stronger antioxidant effect than BSA. Their results for the first experiment showed that the intensity of red color as well as the area of these lesions were inhibited by the topical application of sericin after UVB treatment.

Zhaorigetu and co-workers (2003) also did immunohistochemical analyses to evaluate the level of 4-hydroxynonenal (4-HNE), and the expression of cyclooxygenase-2 (COX-2) protein, and proliferating cell nuclear antigen (PCNA)-labeling index in the UVB-exposed epidermis. 4-HNE is an epidermal oxidative stress marker. COX-2 serves as an early marker of epidermal UVB exposure. Zhaorigetu and co-workers (2003) reported its expression to increase in mice and human skin as well as in human keratinocytes exposed to acute and chronic UVB light. PCNA is an indication of UVB-induced increases in epidermal proliferation. Their results showed that the expression of all three these proteins was significantly suppressed by sericin application.

In their second experiment Zhaorigetu and co-workers (2003) evaluated the tumour protective effect of sericin in hairless mice. The mice were treated with 200 nmol of 7,12-dimethylbenz anthracene (DMBA) and then, 1 week later, were treated with irradiation of 180 mJ/cm^2 of UVB twice weekly for 22 weeks. DMBA is a tumourogenic substance. The protective effect of sericin, at a dose of 5 mg, was evident in terms of significant reduction in tumor incidence and tumor multiplicity. Zhaorigetu and co-workers (2003) finally concluded that sericin has a photoprotective effect against UVB-induced acute damage and tumour promotion by reducing oxidative stress, COX-2 and cell proliferation in mouse skin.

Zhaorigetu and co-workers (2003) showed that sericin has protective effects in first degree burns such as sunburn. The question then arises whether silk proteins such as sericin will have a positive effect on partial thickness or full thickness wounds.

Aramwit and Sangcakul evaluated the effect of sericin cream on wound healing in 2007. They tested its effect on 9 Sprague-Dawley rats by creating an excision on the dorsum of each rat. They found that the inflammatory reaction of the wounds treated with the sericin cream was smaller than the control group throughout the study. Their results showed a statistically significant difference in the percentage of wound size reduction between the sericin treated group and the control group. They also evaluated the histology of the wounds. They found that the group of rats treated with sericin showed better epithelialization and collagen deposition. Aramwit and Sangcakul (2007) concluded from their results that sericin has wound healing properties without causing allergic reactions.

A similar study was performed in 2000 by Sugihara *et al.* They studied the effect of a fibroin film wound dressing on full thickness skin wounds in mice. They created dermatotomies on the dorsal wall of each mouse. They found that, within 21 days the wounds treated with the fibroin film dressing showed better recovery than the control group with more epidermal regeneration and a decreased recovery time.

Their histological findings also revealed that the collagen deposition in the treatment group was greater with less inflammation. They concluded that silk based dressings offer advantages over other dressings and may be clinically useful for wound treatment (Sugihara *et al*. 2000).

Fibroin was also evaluated *in vivo* by Yeo *et al.* as part of a PVA/chitosan/fibroin (PCF)-blended spongy sheet. Their experimental animal of choice was Sprague-Dawley rats. They created a 2 x 2 cm wound on the dorsum of each rat by excision of the skin, including the dermis. These wounds were then covered with the PCF sheets. The control group wounds were covered with conventional gauze dressings. After 12 days the wounds were observed and histological samples were taken and evaluated by hematoxylin and eosin staining as well as Masson's Trichrome staining. Their histological evaluations revealed that the treatment group had an absence of inflammatory cell infiltration and an increase in vascular ingrowth. Also

regeneration of the skin around the wound was faster when compared to the control group (Yeo *et al*. 2000).

In this thesis a novel silk protein based wound dressing is evaluated. The novel silk protein based wound dressing contains sericin and fibroin as primary actives. As seen above literature reports that these proteins do indeed have properties that improve wound healing. The novel silk protein based wound dressing also contains trace elements of copper and literature has shown that copper ions also have positive effects on wound healing.

2.4 COPPER FACILITATES WOUND HEALING:

Copper is an important trace element with numerous physiological functions. It affects the activity of many enzymes such as copper/zinc –superoxide dismutase ceruloplasmin, cytochrome oxidase, tyrosinase, dopamine hydroxylase and lysine oxidase. It affects these enzymes by functioning as a cofactor or allosteric component. These enzymes are vital for cellular respiration, defense against free radicals, melanin synthesis, formation of connective tissue and for iron metabolism. Copper is also essential for copper-dependant transcription factors in gene expression.

All cells in mammalian organisms need a sufficient oxygen and nutrient supply in order to survive and develop. The supply of these resources is sustained by the dense network of blood vessels. The diffusion limit for nutrients and inorganic substances is 100–200 mm from the blood vessels. Therefore, cells are located within this limit (Nasulewicz *et al*., 2004). Oxygen and nutrient supply is also very important to sustain wound healing (Streit *et al*., 2000). In wound healing, angiogenesis restores vascular perfusion to damaged tissues and enhances the recruitment of leukocytes to the site of injury (Bernardini *et al*., 2003). Therefore, healing is characterized by the formation of richly vascularized, hyperpermeable granulation tissue (Streit *et al*., 2000).

Granulation tissue normally forms after about 3 to 4 days post-injury. Granulation is so called because of the granular appearance of newly forming tissue. Granulation tissue consists of numerous new capillaries, macrophages, fibroblasts, and loose connective tissue (Clark, 1996). The proliferative stage of wound healing is characterized by the formation of granulation tissue as well as endothelial cell migration and proliferation (Cohen *et al*., 1992; Fonder *et al*., 2008). Copper has been documented by Nasulewicz *et al.* in 2004 to be necessary for endothelial cell activation. It was also documented that copper stimulates their proliferation and migration.

Copper stimulates wound healing by activating angiogenic factors such as VEGF, bFGF, TNF-α and IL-1. These activated angiogenic factors then bind to endothelial cells initiating the change over from the G0 phase of the cell cycle to the G1 phase resulting in endothelial cell proliferation (Nasulewicz *et al*., 2004).

Gupte and Mumper (2009) reported that copper ions also stimulate the motility of endothelial cells *in vitro*. They also stated that copper serves as a cofactor in red-ox reaction of enzymes such as lysyl oxidase that are responsible for cross-linking elastin and collagen. Additionally, they reported that copper salts induce the synthesis of fibronectin *in vitro*. Fibronectin is a matrix glycoprotein associated with angiogenesis.

In a review article Borkow and co-workers (2008) reported that copper is involved in wound healing by promoting angiogenesis by stimulating VEGF expression. They also reported that topical copper sulphate accelerated the closure of excisional murine dermal wounds. It became apparent, after histological analysis, that the treatment also improved the quality of the regenerating tissue. The density of the cells in the granulation layer of copper-treated wounds was higher and the hyperproliferative epithelial tissue was more (Borkow *et al*., 2008; Sen *et al*. 2002). They also report that copper modulates integrins expressed by the differentiated

keratinocytes of the basal layer during the final stages of healing. These integrins play an essential part in wound healing (Borkow *et al.*, 2008; Tenaud *et al.*, 1999). Low levels of copper improves fibronectin stabilization. Copper is also essential for copper-dependant enzymes necessary for cell proliferation and extracellular matrix remodeling (Borkow *et al.*, 2008).

Additionally copper ions have been used as a disinfectant for centuries, including the cleaning of human tissue. Even though copper has been used as a disinfectant for so long, there are still very few copper tolerant microbes (Borkow *et al.*, 2008; Borkow and Gabbay, 2005). The importance of copper in wound healing is further confirmed by the positive effect of its administration in cases of severe burn trauma in children as well as in the management of phosphorus burns (Borkow *et al.*, 2008).

According to literature it is clear that both silk proteins and copper ions have a positive effect on wound healing. In order to evaluate the effect of the novel silk protein based wound dressing, that combines these two elements, an appropriate animal model needs to be chosen.

2.5 BURN WOUND MODELS:

A model is defined as a scaled reproduction or representation of a process which may be static or dynamic (Mani *et al.*, 1999). A wide range of animal models have been used in various studies in order to simulate complex biological processes occurring in humans (Mani *et al.*, 1999; Fang *et al.*, 1997). Such stimuli include surgical, pharmacological, mechanical and even physiological stimuli.

In health care, impaired wound healing has led to considerable morbidity and mortality. For this reason it has been one of the most intriguing areas in biomedical research for some time. Animal models serve this requirement and have long been fundamental for not only studies concerned with wound healing but also other medical and scientific research (Mani *et al.*, 1999). These studies include those

concerned with the treatment of burn wounds, investigating burn wound pathology, local therapy, the influence of systemic drug application on the burn wound and the effect of burn trauma on the organism as a whole (Knabla *et al*. 1999).

The apparent abnormalities presented in animal experiments seem to be a reflection of the unique biological phenomenon of the particular species being studied. However, it could also be a result of the unnatural means by which the disease was induced or it could simply be due to the stressful environment of the laboratory. It is very important to remember that the process of stressing to induce changes that mimic disease, in this case wound healing, will limit the applicability of the results. In addition, it is well documented that human skin is unique and highly variable according to age, sex, race and region of the body. There is no single model that can fully represent the true biological occurrence in human tissues. It is therefore important to keep in mind the inherent limitations in using animal models. The knowledge obtained from animal studies cannot be transcribed, without reservation, to humans. The differences in morphology and function must be fully understood before any conclusions can be drawn (Mani *et al*., 1999).

However, it is not always possible to use human tissue for experimental purposes, for this reason an appropriate animal model can be used (Freshnee, 2005). Although there are many differences, humans and animals share simple basic structures and patterns of cell proliferation and differentiation (Mani *et al*., 1999).

Rodents that have been used in burn research include, but are not limited to, guinea pigs, Sprague Dawley rats, Wistar rats, Long Evans rats and gold hamsters. Another rodent animal model that has also shown to be useful in burn studies is the rabbit, for example, New Zealand white rabbits. Although rodents have been used in various animal studies concerned with burn wounds, the preferred animal in burn experiments is the pig. The porcine skin is hairless and very similar to human skin. Unfortunately pigs pose practical problems as research subjects. They need more

housing space and are more expensive to maintain than smaller animals (Knabla *et al*. 1999).

2.5.1 The porcine burn wound model:

Pig skin is anatomically and physiologically more similar to human skin than that of other animal models. Porcine models are ideal for examining therapeutic agents for wounds. Results from previous studies have shown that porcine models are more comparative to human studies. There are several established porcine models for wound healing studies. In some models the wound is created by an excisional method, this allows for a more controlled wound. However, this method leads to an outcome with a different pathophysiology than that seen in burn wounds. Other models have several wounds on the same side of the animal, but this could be undesirable as the systemic effects of some agents could mask some of the results (Cuttle *et al*., 2006).

In 2006 Cuttle and co-workers (2006) used juvenile Large White pigs to develop a reproducible model of deep dermal partial thickness burn injury. Their aim was to create an animal model which mimics the human situation. An animal model which demonstrates the pathophysiology of a burn and forms a hypertrophic scar was needed in order to study the processes of the burn wound healing response and hypertrophic scar formation.

Deep dermal partial thickness burns take longer than 2–3 weeks to heal. These type of burn wounds heal with contracted, hypertrophic scars that require long-term scar management. Using a porcine animal model they where able to create larger burns needed to study and to represent a significant burn, while still being ethically acceptable and without inducing major systemic responses in the animal.

The drawback of most models is that the burns created in these models are quite small, ranging from 6 cm^2 to 19 cm^2. Cuttle and co-workers (2006) were able to create a good, reasonably large contact injury that was uniform. This wound was

about 50 cm^2. They achieved this with a device that was able to conform to the shape of the porcine side.

When studying hypertrophic scars, a tight skinned model was required. Loose skinned animals such as rodents and rabbits do not physiologically form hypertrophic scars. The juvenile female red Duroc pig, has been reported to be a good model for this application. Duroc pigs have been shown to reliably develop contractile, fibroproliferative scars. Additionally, these scars are characterized by more than 80% wound contraction. Also, thickening of the skin layers namely the stratum corneum, epidermis and dermis, and excessive, disorganized collagen deposition and collagen nodule formation are characteristic of this model (Gallant-Behm *et al*., 2006).

Juvenile female Yorkshire pigs have also proven to be a good model for wound healing. Full thickness wounds in juvenile female Yorkshire pigs heal similarly to normal human wounds. This healing process is characterized by modest wound contraction, and the formation of a normal, mature scar (Gallant-Behm *et al*., 2006).

Pigs are only one of the many animal models used for studying burn injury and scar formation (Cuttle *et al*., 2006). Sheep have also been used in the study of burn wound healing.

2.5.2 Sheep burn wound models:

One of the advantages to using a larger animal model for burn studies is that a larger body size allows a larger burn to be created. Larger burn wounds are generally easier to study. The disadvantages are that these size animals are not as readily available as rodents, are expensive and expensive to house. Additionally such large animals are subject to much more strict legislation. Also, literature on sheep animal models is not always available and the method of anesthesia and euthanasia is much more complex and expensive (Martin, 1994). Another disadvantage of lamb ovine burn models, is that sheep skin produce lanolin and

wool that are unlike human skin. This characteristic also makes topical application of treatments difficult (Cuttle *et al*., 2006).

In 2001 Sakurai and co-workers used the sheep as a burn wound model. Their reasoning for using this model was that it would allow them to measure systemic hemodynamic alterations. According to Sakurai and co-workers (2002) this model also mimics both systemic and local responses seen in severe burn patients. Additionally, Sakurai and co-workers (2002) provide information of microvascular alterations in the tissues below the burn, as well as the surface skin.

Even though these larger animals, such as pigs and sheep, are good models to study the healing processes of burn wounds, the best model would be a human model.

2.5.3 Human burn wound models:

In the literature mentioned above various good animal models for studying burn wounds where discussed. All of these models have one disadvantage in common: none of them are human (Freshnee, 2005). These models are easier to standardize and sample but they differ from humans with respect to wound healing processes. For this reason the results have to be substantiated in burn wounds in human skin. The only true representative of a healing burn wound in human skin is human skin itself. Only human skin can truly reflect the events that lead to healing of a human burn wound.

The disadvantage when studying human wounds, is that human wounds *in vivo* are inconsistent and difficult to sample. Additionally, in clinical practice, each patient's healing process is influenced by several factors that vary between patients. These factors include the nutritional status of the patient, age, circulation, infections and the initial treatment of the burn wound. Therefore, when studying either the local occurrences and events involved in the healing of a human burn wound, or the effects of treatments such as pharmacological agents applied to the wound, a well

controlled *in vitro* model could be useful. This is especially true in an *in vitro* model with a large number of burn wounds with standardized depth and area.

Models have been developed to provide standardized ways to study responses to different stimuli in different cell types. One example is the *in vitro* wound healing models that involved mechanical lesion in cell monolayers. Another model involved a living skin equivalent which is made up of a collagen gel that contains fibroblasts on which cultured keratinocytes are plated.

The problem with models such as these is that they are artificial compositions of cultured cells in which not all of the cell types and biochemical factors involved in the wound healing process are present. Unfortunately, these models are not only limited but possibly also misleading. It doesn't give an accurate representation because of the complex interactions between different cell types during the wound healing process *in vivo*.

The healing of full-thickness human skin wound explants *in vitro* has been described previously by Kratz and co-workers in 1994. The study by Emanuelsson and Kratz (1997) developed a standardized and easily reproducible *in vitro* model for the healing process of human skin burn wounds. Human skin explants were obtained and full-thickness burn wounds were created in a standardized way. These explants were then stimulated to heal by incubation with fetal calf serum. The viability of the cells in the dermis and epidermis of the explants was determined and the histology of the burn wounds was studied throughout the re-epithelization phase.

They found that both keratinocytes and fibroblasts present in the explant were viable after 2 weeks of incubation in at least 2% fetal calf serum. Additionally, the wounds incubated in 10% fetal calf serum showed to be completely re-epithelialized after 7 days. In contrast, the wounds incubated in 2% fetal calf serum did not show any sign of healing, even though cells in the dermis and epidermis were shown to

be viable. Their model allowed the exclusion of systemic factors such as circulation, nutrition and the inflammatory response. Excluding these factors made it easy to control and measure the healing processes of the wounds. Furthermore the model is reproducible and easy to perform.

They claim that the *in vitro* model presented in their study includes all the cells and matrix components that are present in the wound healing area *in vivo*. They also claim that because their model excludes systemic variables, it provides a way to study local responses such as the auto-paracrine interactions between different cell types in the burn wound area. As with most models it is important to consider systemic factors when the results are transferred from the *in vitro* model to the *in vivo* situation. This is especially true for their model.

Another aspect to consider is that their study is limited to the re-epithelialization stage of the burn wound. However, they claim that due to the possibility of keeping the cells in the explant viable over an extended period of time points out that the model may be important in studies of other stages in healing, such as cell differentiation and production of matrix components. They also found that viable, proliferating fibroblasts were present in the wounded explants after 14 days of incubation. This finding suggests that there is an ongoing formation of granulation tissue in the wounded dermis therefore their burn wound model can possibly be used to study the important granulation phase of the healing of a burn wound.

In addition this *in vitro* model, according to Emanuelsson and Kratz (1997), has potential applications for studying skin grafting on burn wounds such as cultured autologous keratinocyte grafts. However, in order to study burn wound healing *in vivo*, rodents have been used most often.

2.5.4 Rodent burn wound model:

As previously mentioned many different rodents have been used in previous burn wound studies. These models include guinea pigs, Sprague Dawley rats, Wistar rats, Long Evans rats and gold hamsters. Another rodent-like animal that has been used in burn wound studies is the rabbit.

In rabbits the structural arrangement of the skin layers is similar to that of human skin. Between humans and rodents, there are a few differences. These differences lie in the thickness of skin layers, the distribution of hair follicles and the distribution in sweat and sebaceous glands. A significant difference between the skin of rabbits and human skin is that rabbit skin is more elastic than human skin. However, this difference does not interfere with the usefulness of this model to study certain aspects of the burn wound such as burn depth (Knabla *et al*. 1999).

Humans and rats also share characteristics with regards to skin. These characteristics include the presence of an epidermis, a basement membrane, hair follicles and a dermis. Clearly there are a number of differences with regards to anatomy and physiology. In humans the skin adheres tightly to the underlying tissue while rat skin is looser, but the most significant difference is that the rat skin contains a panniculus carnosus which is a subcutaneous layer of muscle tissue (Martin, 1994).

The advantages to using a rodent as an animal model for studying burns are that they are readily available and relatively inexpensive. There is also a large amount of literature using rodents as burn models. The major disadvantage to using a rodent animal model is that they have a small body size thus relatively small burn wounds can be produced (Mather and Roberts, 1998).

Despite the disadvantages, many studies use small animals such as rodents, especially rats (Cuttle *et al*., 2006). After considering the advantages and

disadvantages of the various possible animal models reviewed in the literature, the rat burn wound model would be the most appropriate model for the current study.

2.6 RESEARCH QUESTIONS:

The literature reviewed clearly shows that sericin, fibroin and copper ions have healing properties. Therefore, a burn wound dressing containing these actives such as the novel silk protein based wound dressing may prove to be beneficial for burn wound healing. The following research questions will therefore be investigated in the current study:

- Can the Wistar rat be used to establish a burn wound protocol mediated by a novel silk protein based wound dressing?
- Is an extract prepared from the novel silk protein based wound dressing cytotoxic?
- What is the effect of the novel silk protein based wound dressing on granulation and epithelailization after application to a burn wound?
- What is the effect of the novel silk protein based wound dressing on collagen deposition after application to a burn wound?
- What is the effect of the novel silk protein based wound dressing on the systemic white blood cell counts in blood smears after application to a burn wound?
- Will the novel silk protein based wound dressing have an impact on fibrin network architecture in circulating plasma, and does a local burn injury treated with the novel wound dressing change clot ultrastructure?

Chapter 3: The cytotoxic effects of a novel silk protein based wound dressing on human fibroblasts in primary culture

3.1. INTRODUCTION:

Wound healing consists of three stages: the first stage is involved with the cleansing of the wound, followed by granulation and vascularization and finally epithelialization. These stages require an optimum microenvironment and the absence of any cytotoxic factors (Paddle-Ledinek *et al.* 2006).

In vitro assessment is the study of the effect of a substance or material in cell culture. This type of study is the precursor to an animal study. There are two different kinds of cell cultures: the primary cell culture and the permanent cell culture. Permanent cell cultures have an unlimited proliferation capacity; cultures such as these are the HeLa (human cervical adenocarsinoma cells) cell line. Permanent cell cultures such as the HeLa cell line originate from tumours or transformed cells. Primary cell cultures originate directly from an organism and can maintain the differentiated state only for a short period of time, thus they have a limited lifespan. A primary cell culture is that stage between isolation of the cells and the first subculture.

According to the fifth edition “Culture of Animal Cells: A manual of basic technique”, when choosing an appropriate cell type for culture one has to take into consideration whether the species is important, the phenotypic expression and whether a finite or continuous cell line would be most applicable. In permanent cell cultures certain characteristics are lost such as anchorage dependence, contact inhibition and density limitations of cell proliferation. Therefore, a primary cell culture would be a better representation of the natural state (Freshnee, 2005). Fibroblasts, keratinocytes, macrophages and endothelial

cells are the cells that are most often investigated in wound healing research (Gottrup *et al.*, 2000). Since fibroblasts play a central role during the healing process (Cohen *et al.*, 1992), they would be the obvious choice for a study aimed at examining the effects of a novel silk protein based wound dressing on cells in culture. The ultimate aim is to use this specific wound dressing to treat wounds in humans. The best applicable cell culture to use would therefore be a human fibroblast primary cell culture.

The novel granulation promoting wound dressing has two active components, copper ions and silk derived proteins. Therefore it is necessary to review the literature on the effect of these two components in cell culture.

3.1.1. Copper:

Systemic copper is involved in many areas of wound healing. According to Sen and co-workers (2002) copper sulfate clearly promotes angiogenesis, *in vitro* and *in vivo*, through vascular endothelial cell growth factor (VEGF). Copper-treated wounds have more prominent VEGF expression, as seen in immunohistochemical studies of the wound edges (Sen *et al.* 2002). Prudovsky and co-workers (2003) concluded that the release of the pro-angiogenic polypeptides, fibroblast growth factor-1 (FGF-1) and IL-1α is dependant on the availability of copper ions. Also, when Tenaud and co-workers (1999) studied the *in vitro* modulation of keratinocyte wound healing integrins by zinc, copper and manganese they found that in basal layer keratinocytes copper modulates integrins expression, which is an essential part during the final stages of wound healing. According to Lansdown (2002), in highly mitotic regions such as wound margins, application of copper upregulates the expression of the mattalothioneien gene. The action of mettalothioneiens may be a result of the large number of copper-dependant enzymes involved in cell proliferation and matrix remodeling. Literature suggests that copper may also improve tissue remodeling through the enzyme metalloproteinase-2 (Maquart *et al.* 1993; Canapp *et al.* 2003; Simeon *et al.,* 2000; Borkow *et al.* 2008).

Nasulewicz and co-workers (2004) also state that copper is an essential trace element necessary for various physiological functions. Copper acts as a co-factor, an allosteric component and is important for copper-dependant transcription factors. They also state that copper plays an important role in angiogenesis. It is required not only for endothelial cell activation but also stimulates their proliferation and migration. Angiogenesis is a central component of granulation tissue formation (Clark, 1996). Therefore it can be hypothesized that copper may also enhance granulation tissue formation. However, copper has been reported to be cytotoxic at certain levels (Kawakami *et al.*, 2008).

In 2008 Kawakami *et al.* studied the mechanism of apoptosis induced by copper in a rat pheochromocytoma clonal cell line (PC12). In order to determine the cytotoxicity of copper, they studied cell viability using the trypan blue exclusion assay. PC12 cells were exposed to copper concentrations ranging from 0 to 200 µM for a period of 48 hours. The negative control cells were cultured under apoptotic conditions, meaning serum-free medium. PC12 cells exposed to 0.02 µM and 2 µM copper concentrations showed a significant increase when compared with the negative control. The cells cultured in the maximum concentration (200 µM) of copper showed viability similar to the PC12 cells cultured under apoptotic conditions. This indicates that copper is cytotoxic at higher levels and that copper cytotoxicity increases as the copper concentration increases (Kawakami *et al.,* 2008).

3.1.2. Silk derived proteins:

Silk is derived from the silkworm, *Bombyx mori* (Zhang, 2002). In its natural form silk is composed of a filament core protein know as silk fibroin, and a glue-like coating of sericin proteins (Wang et al. 2006a). Silk sericin has been documented to have various characteristics that would be beneficial in a wound dressing. Terada and co-workers (2002) reported that hydrolyzed sericin can be used as a supplement for cell culture media to accelerate the proliferation of mammalian cells.

Sericin has also been documented to enhance attachment of cultured human skin fibroblasts. Tsubouchi and co-workers (2005) revealed in their study that when human skin fibroblasts are cultured on Petri dishes coated with sericin, the cell count was 250% of the non-sericin coated control after 72 hours. Besides these properties sericin has also been documented to have antibacterial, anti-oxidant and UV-light protective activity (Aramwit and Sangcakul, 2007).

Yang and co-workers (2007) conducted a study evaluating the biocompatibility of silk fibroin with peripheral nerve tissue and cells *in vitro*. They conducted an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide] analysis and found that the viability of Schwann cells cultured in silk fibroin extract was not significantly different from the control. They concluded that silk fibroin supports the cell growth of dorsal root ganglia and facilitates the survival of Schwann cells without significant cytotoxic effects on the cell phenotype or cell functions. Even though the cytotoxicity of silk proteins and copper has been evaluated individually, it has not been evaluated in combination. It is therefore necessary to asses the cytotoxic effects of an extract prepared from the novel silk protein based wound dressing.

3.2. MATERIALS AND METHODS:

3.2.1. Novel silk protein based wound dressing:

The novel silk protein based wound dressing was supplied by Southern Formulations (Pty) Ltd.

3.2.2. Preparation of the extract:

A sample sized 2 x 2 cm was cut from the novel silk protein based wound dressing. The sample was placed in a 15 ml tube containing 5 ml of medium. The 15 ml tube containing the sample in medium was placed in the incubator for 24 hours to

dissolve. The concentration was thus calculated to be 0.8 cm^2 of dressing per ml of medium.

3.2.3. Media, supplements and reagents:

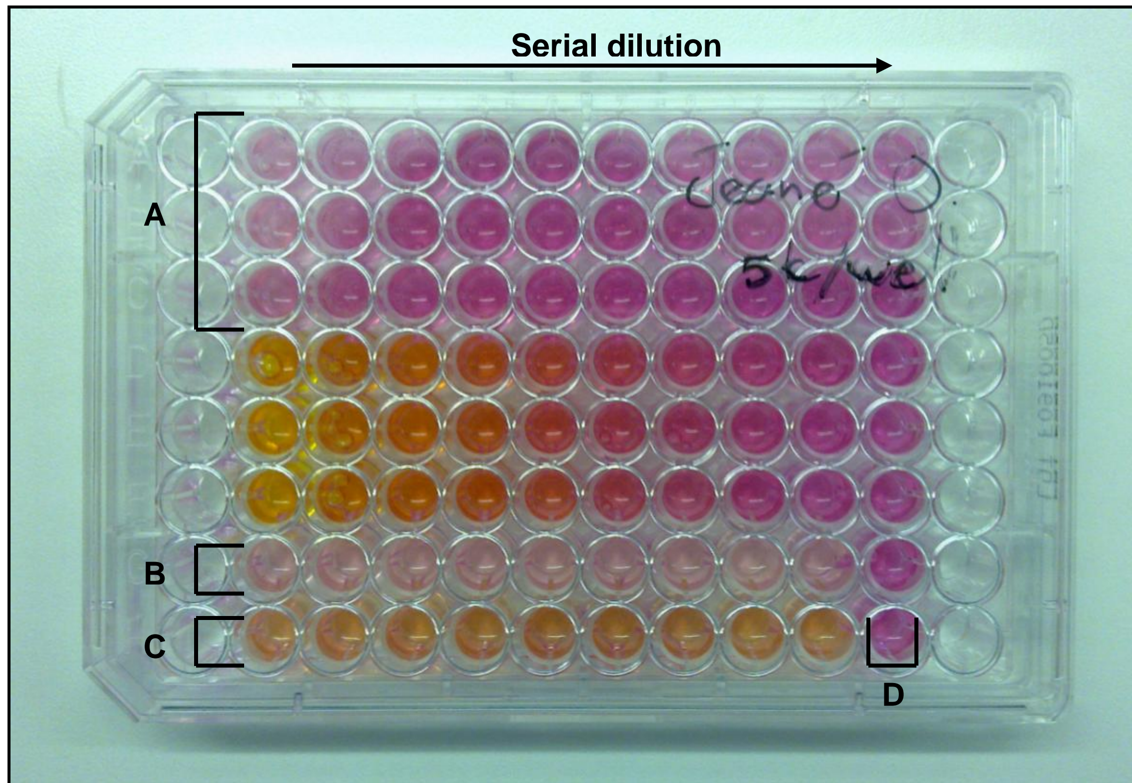
MTT and medium supplements were bought from Sigma Aldrich, Atlasville, South Africa. 96 well plates, 75 cm^2 cell culture flasks, 5 ml and 10 ml pipettes, 15 ml and 50 ml centrifuge tubes and eppendorf tubes were purchased from Lasec supplied by Labotec, South Africa. Media was obtained from Whitehead Scientific, South Africa. All glassware was sterilized by Glycar South Africa using autoclave steam sterilization. Distilled sterilized water was also purchased from Glycar South Africa.

3.2.4. Human fibroblast primary culture cells:

Human fibroblast primary culture cells were supplied by Southern Formulations (Pty) Ltd. All ethical implications where resolved by the supplier. The cells were plated at a cell concentration of $5x10^3$ cells per well in a 96 well plate and were kept at 37°C and 5% CO_2 for 24 hours before exposure to the extract.

The cells were exposed to a series of dilutions ranging from 25 mg/µl to 0.02 mg/µl for 24 hours. See figure 3.1. Exposure to the extract was performed in triplicate.

Figure 3.1: Photograph showing a 96 well plate containing fibroblasts exposed to serial dilutions of the dressing extract (**A**). **B** indicates the positive control and **C** the negative control. **D** indicates the control group not exposed to the extract.



3.2.5. The MTT Assay:

The MTT assay measures cell viability. The principle of the MTT assay is based on the ability of the mitochondrial dehydrogenase enzyme, active in viable cells, to cleave the yellow tetrazolium rings to produce blue formazan crystals. In this manner the colour intensity can be measured and is an indication of cell viability and also of cell proliferation, if any.

In each well a 100 µl of culture medium was replaced with a 5% MTT solution in phosphate buffered saline. The cells were then incubated at 37°C and 5% CO_2 for 3 hours. The MTT solution was then replaced with 100 µl DMSO (Dimethyle sulfoxide) in order to dissolve the blue formazan crystals. After shaking the plates for 20 minutes the absorbance was measured at 450 nm with a Bio-Tek (model

ELx800) microplate reader (Pariente *et al*. 2002; Cetin and Bullerman, 2005; Huveneers-Oorsprong *et al*. 1997).

3.2.6. Statistical analysis:

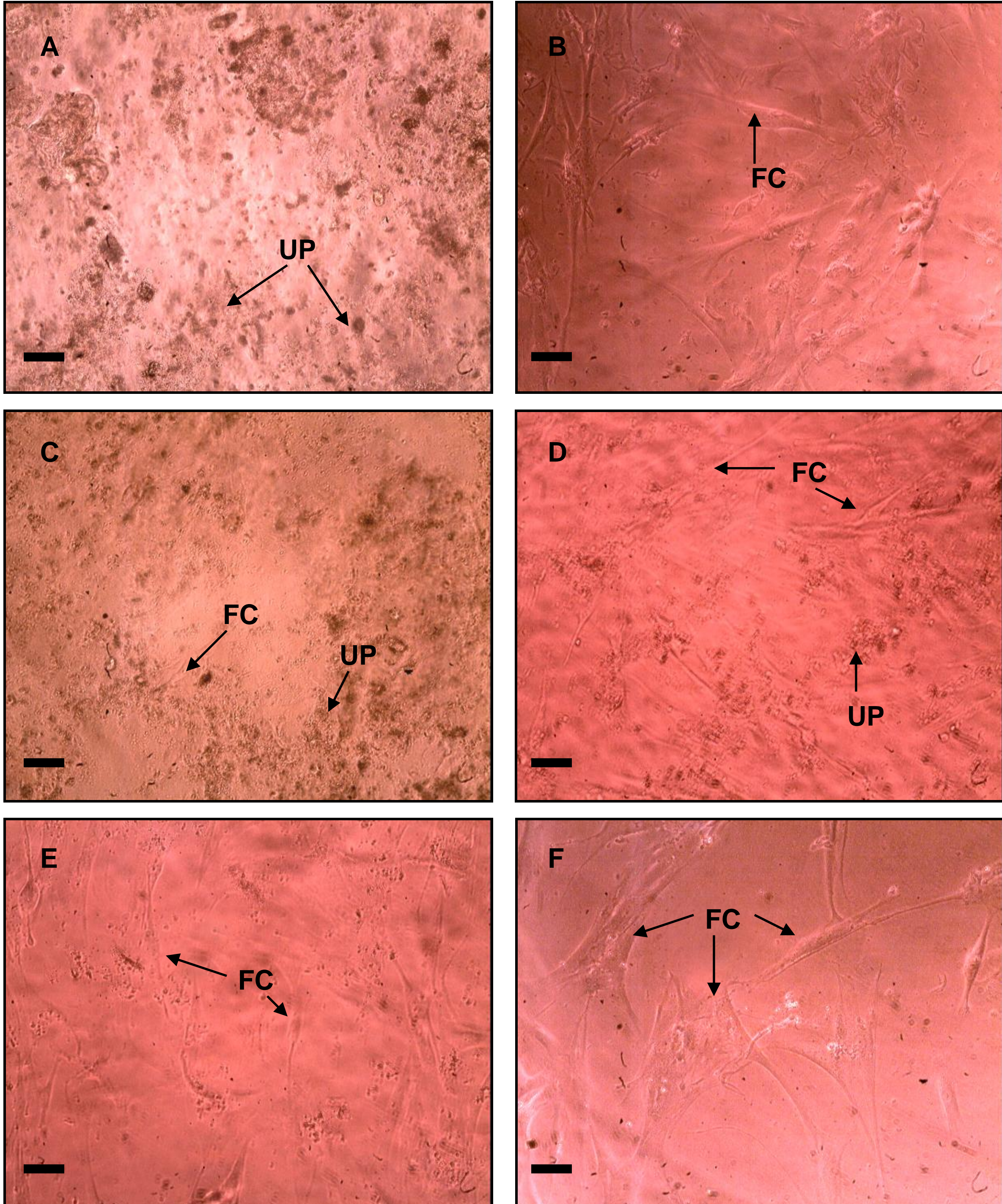
The experiment was done in triplicate for each concentration; additionally the experiment was repeated three times. Statistical significance was determined at $p < 0.05$ using ANOVA (www.graphpad.com).

3.3. RESULTS

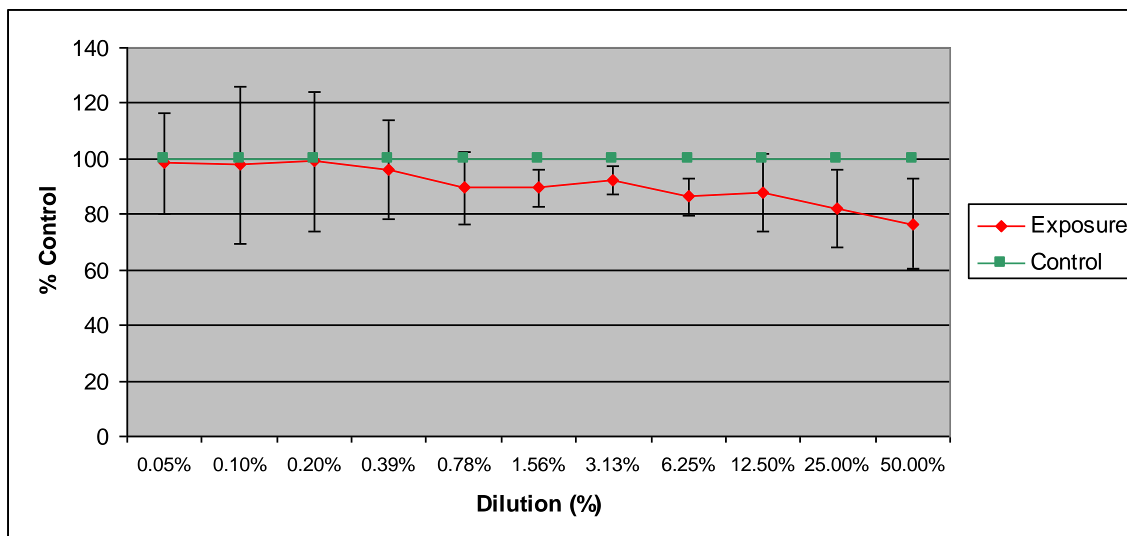
Figure 3.2 shows the extract with some undissolved particles (**UP**) present. These particle are also present in the medium of the exposed cells, however the particles become less as the extract is diluted. This is expected. Figure 3.2 also shows the control cells not exposed to the extract. Fibroblast cells (**FC**) are clearly visible. Figure 3.2 also shows the fibroblast cells in culture exposed to different dilutions of the extract. See Figure 3.2 **E** to **F**

Graph 3.1 shows the results of the cytotoxic analysis after absorbance was measured at 450 nm. The MTT assay measures cell viability based on mitochondrial activity. The graph reveals that there is a proportional decline in cell viability as the concentration increases. Therefore cell viability is inversely proportional to extract concentration. This is similar to the results found by Kawakami and co-workers (2008) on PC12 cells. The highest concentration, 50% extract or 25 mg/µl, shows a cell viability of 75.3% of the control. The graph does not show additional cell proliferation at any concentration. At the lowest concentration, 0.05% or 0.02 mg/µl, the cell viability is 95% of the control.

Figure 3.2: Photographs showing the dressing extract (**A**) and the control cells that are not exposed (**B**). Undissolved particles form the dressing (**UP**) are visible as well as fibroblasts (**FC**). Photographs of the fibroblasts in culture exposed to 50% (**C**), 12.5% (**D**), 3.13% (**E**) and 0.78% (**F**) of the extract are also shown.
Scale = 20µm.



Graph 3.1: The effect of a serial dilution of dressing extract prepared in cell culture medium on human fibroblast cell viability after 24 hour exposure.



3.4. DISCUSSION

This study was undertaken to evaluate the cytotoxic effect of an extract prepared from the novel silk protein based wound dressing.

Morphological evaluation of the cells exposed to different dilutions of the extract did not show any signs of apoptosis at any concentration. The morphology of the exposed cells did not differ from that of the control. See figure 3.2. The effect of the extract on cell viability was measured using the MTT assay.

The MTT assay measures cell viability. The principle of the MTT assay is based on the ability of the mitochondrial dehydrogenase enzyme, active in viable cells, to cleave the tetrazolium rings to produce formazan crystals. Tetrazolium rings are a pale yellow colour, water soluble and able to penetrate the cell membrane while the formazan crystals are blue, water insoluble and not able to penetrate membranes. The formazan crystals that form as a result of the activity of the mitochondrial dehydrogenase enzymes accumulate in viable cells. The colour formation can then be quantified with a scanning spectrophotometer. The

intensity of the blue colour achieved is therefore directly proportional to the metabolic activity of the cells in culture and inversely proportional to the toxicity of the extract (Pariente *et al*. 2002; Cetin and Bullerman, 2005; Huveneers-Oorsprong *et al*. 1997).

Even though a decline in cell viability is observed no IC_{50} level was reached up to a concentration of 25 mg/µl. IC_{50} is defined as the concentration of extract required for 50% inhibition of cell proliferation or in this case inhibiting MTT reduction by 50% (Cetin and Bullerman, 2005; Kania *et al.,* 2007; Huveneers-Oorsprong *et al*. 1997).

Additionally, the graph indicates no increase in cell proliferation, even at lower concentrations. One of the disadvantages of a primary cell culture is that the growth rate is slower than that of a permanent cell line. The doubling time is documented to be 24 to 96 hours (Freshnee, 2005). When analyzing the cytotoxic effects in this study, the cells where cultured for an incubation period of 24 hours. In order to assess proliferation rate; it would be necessary to allow cell growth for a longer period of time, therefore a longer incubation time.

3.5. CONCLUSION

The results indicate that the novel silk protein based wound dressing is not cytotoxic at the concentration levels tested. No IC_{50} level could be found. Literature suggests that copper ions could be cytotoxic at higher levels. Therefore, further testing would be necessary to determine at how high a concentration the dressing extract could be cytotoxic.

It is known that both active components, silk proteins and copper ions, can be beneficial for cell proliferation. However, the results didn’t indicate any additional proliferation. It is also known that primary cultures have a relatively slow growth rate with a doubling time of 24 to 96 hours. Therefore, it is expected that additional proliferation would not be seen in this study. However, by extending the incubation

period this effect on cells in culture could be seen. However, the objective of this study was to evaluate cytotoxicity and not the effect on proliferation.

Additionally, a cell culture study only studies the effect of a substance on that particular cell type. It does not take into consideration the other environmental factors such as cytokines from other cell types. In order to study the full effect of this dressing on granulation and epithelialization in particular, an *in vivo* study, as done for this thesis, would give a better indication.

Therefore, in conclusion, the novel silk protein based wound dressing is not cytotoxic at concentrations ranging between 0.02 mg/µl and 25 mg/µl. Also, the novel silk protein based wound dressing does not positively affect cell proliferation of a human fibroblast primary cell culture within 24 hours of exposure. Further studies can be done to study the effect of the novel silk protein based wound dressing on cell proliferation in future.

Chapter 4: Implementing the burn wound model and determining the effectiveness of Butorphanol as part of the analgesic regimen

4.1. INTRODUCTION:

In some wound healing studies, especially in those individuals with impaired wound healing ability, ethics prohibit the use of humans. In order to replicate wound healing in humans, animal models are commonly used in wound research. Wound healing is a complex process consisting of various stages that involves cell and extracellular matrix interactions that can affect tissues and organ systems. One method of studying wound healing is by use of an *in vivo* model.

There are numerous advantages to using rats as a skin wounding model. Rats are readily available, and have a more economical and efficient use of laboratory space and housing facilities due to it's relative small size and low cost. Another important advantage is the availability of animals with well-defined health and genetic backgrounds as well as a large collection of literature documenting biological responses and parameters for rats (Dorsett-Martin and Wysocki, 2008).

One of the well documented biological responses is that age has a significant influence on the rate of wound healing, resulting in a prolonged or poor healing process. Age-related differences in wound healing have not only been evident in rats but also other animal models. Additionally, rat ages and body weights can be interrelated. Rats within the age group of 3 to 6 months are comparable to young adult humans (Mogford and Mustoe, 2001; Dorsett-Martin and Wysocki, 2008). This makes rats of about 250g ideal for burn wound studies eliminating variables in wound healing regarding age.

Another advantage to using rats as a wound healing model is that the wound healing process in rats is much quicker than that of humans making it possible to complete studies evaluating wound healing in a matter of days, instead of weeks. Additionally, it is possible to standardize the type, size, shape and depth of the wound allowing for better comparison of data within and between studies (Dorsett-Martin and Wysocki, 2008).

Humans and rats share characteristics with regards to skin; these include the presence of an epidermis, a basement membrane, hair follicles and a dermis. Clearly there are a number of differences with regards to anatomy and physiology. In humans the skin adheres tightly to the underlying tissue while rat skin is looser. The rat skin contains a panniculus carnosus which is a subcutaneous layer of muscle tissue (Gottrup *et al.*, 2000; Dorsett-Martin and Wysocki, 2008).

Human skin, on the other hand contains apocrine and eccrine glands that rat skin does not. The hair growth pattern also differs between humans and rats. In humans the hair growth follows a mosaic pattern while in rats hair growth occurs in patches. Another difference is the rat's ability to synthesize its own vitamin C while humans do not posses this ability. Also, rats do not form keloids or hypertrophic scars as seen in some humans (Mogford and Mustoe, 2001; Dorsett-Martin and Wysocki, 2008).

Even though there are differences in the structure of the skin, both the human and the rat healing process follow the same stages of healing. The normal mammalian response to cutaneous injury is firstly inflammation, then the formation of granulation tissue followed by epithelialization and remodeling of the newly formed tissue (Dorsett-Martin and Wysocki, 2008).

Epithelialization is the primary response in burn wounds, abrasions and partial thickness wounds. In these wound only the epithelium and superficial dermis are missing. Epithelialization occurs as epithelial cells move from the hair follicles and

sweat glands left in the remaining dermis as well as from the wound edges. In full-thickness wounds there are no hair follicles or sweat glands present in the remaining tissue therefore epithelial cells only migrate from the wound edges and epithelialization occurs at a rate of 1 – 2 mm per day. This applies for both human and rat models (Mogford and Mustoe, 2001; Dorsett-Martin and Wysocki, 2008).

In all wound healing models the analgesic used is of primary importance when considering the ethical implications, especially in burn wound studies. Butorphanol has been documented to elicit good to excellent pain relief in humans (Pachter and Evens, 1985). Butorphanol, on its own and in combination with morphine, has also been documented to be effective for burn wound studies using rats as the animal model (Osgood *et al.*, 1995).

Butorphanol is a synthetic opoid that produces its analgesic effects mainly by acting on kappa opoid receptors, (Fan *et al.*, 2003) however Butorphanol has both agonist activity at sigma and kappa receptors and antagonistic properties at mu receptors (Hedenqvist *et al.* 2000).

According to Osgood and co-workers (1995) both Butorphanol and Morphine are equipotent antinociceptive agents. These two opoids were compared by doing the rat tail withdrawal test and the acetic acid writhing test. In the rat tail withdrawal test Butorphanol showed to be equally as potant as Morphine and in the writhing test Butorphanol showed to have approximately 3.5 times the antiwrithing activity of Morphine on a molar basis. However, Thakore and co-workers (2009) published in their article that Butorphanol is 4 to 8 times more potent, as an analgesic, than Morphine. Additionally Butorphanol exhibits fewer side effects.

In a study conducted by Horan and co-workers (1989) on rats, they showed that Butorphanol had no effect on respiratory rate, while Morphine decreased the respiratory rate. Both Butorphanol and Morphine produced hyperthermia after acute dosing (Horan and Ho, 1989). Also, in humans Morphine has a stronger sedative

effect than Butorphanol (Masica *et al*., 2007). No data regarding the sedative effect is currently available for rats.

Even though Butorphanol has been documented to have better analgesic properties with fewer side effects, Morphine is the most common and most often used analgesic world wide (Andersen *et al*., 2003). Morphine is a well-known and better documented antinociceptive agent than Butorphanol (Masica *et al*., 2007).

For the reasons stated above it is suspected that Butorphanol would be the better choice as an analgesic for a burn wound study such as described in the current thesis. The literature suggests that a large number of burn wound models have been established and used in various burn wound studies. However, very few of these articles give a clear outline of the analgesic regimen used, especially concerning Butorphanol. In order to study the effect of a novel granulation promoting dressing on wound healing, the animal model needs to be established, and this includes the choice of analgesic regimen.

4.2. MATERIALS AND METHODS:

4.2.1. Implementing the burn wound model:

Nine female Wistar albino rats weighing between 200 – 250g, maintained at the University of Pretoria Biomedical Research Centre (UPBRC) were used in this study. The rats were kept in a room with a 12h light and dark cycle and a room temperature constant at 22 °C. The rats were fed a standard rat diet, water *ad libitum* and were housed in individual polycarbonate cages. The rats were fasted for 12h before thermal injury; but they had free access to water. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee (UPAUCC).

This study was divided into two parts. The first part was concerned with the analgesic regimen comparing Morphine with Buterphanol. Three rats received

Morphine and another three rats received Buterphanol. It is considered unnecessary and unethical for this study to have a negative control group.

The second part of this study was concerned with the effect of a novel silk protein wound dressing on wound healing. All six rats in this part of the study received Buterphanol as part of the analgesic regimen. One group of three rats received treatment with a novel silk protein based wound dressing. The remaining three rats were not treated with the novel silk protein based wound dressing. This was the control group. Therefore the rats were divided as follows:

Table 4.1: Summery of the division of the experimental rats.

	No additional treatment	**Treatment with a novel silk protein dressing**	**Total number of animals**
Buterphanol	3 rats (Control)	3 rats	6 rats
Morphine	0 rats	3 rats	3 rats
Total	3 rats	6 rats	**9 rats**

4.2.2. Thermal injury

On the first day (day 0), the rats were anaesthetized with Isoflurane and directly afterwards the analgesic, Tramadol (10 mg/kg), was injected intramuscularly. After injection of the analgesic, 15 minutes was allowed before wound creation in order to allow the analgesic to take effect. The dorsum of each rat was shaved and then exposed to a 1 x 1 cm brass block for 10 seconds (Gottrup *et al.*, 2000; Dorsett-Martin and Wysocki, 2008). This brass block was heated up to 95 °C using a hot water bath. Using gravity only, the brass block was rested on the dorsum of the rats, which resulted in partial thickness skin burns (Mogford and Mustoe, 2001; Dorsett-Martin and Wysocki, 2008). A physiological saline solution was then administrated intraperitoneally (25 mg/kg) in order to prevent dehydration of the animal.

4.2.3 Treatment

Immediately after thermal injury all wounds were dressed with a gauze primary dressing and Opsite® as the secondary dressing. In order to keep the rats from interfering with the healing process the secondary dressing was further covered with a third dressing of bandage and fastened with Elastoplast® around the edges.

On day 3 post-wound creation the rats were divided into two groups. During the dressing changes one group of three rats received the normal dressing changes composed of gauze and Opsite®. This was the control group. Another group of six rats received the normal dressing change composed of gauze and Opsite®. However, their wounds were treated with an additional novel silk protein based wound dressing.

4.2.4. Analgesics

All nine rats received Tramadol *ad libitum* via their drinking water; 50 mg (six capsules) to 45 ml of water with added dextrose (5 ml). The dextrose was added to better the taste of the Tramadol solution. Water intake was monitored by weighing the water bottles at regular intervals. This solution was already given the day before in order for the animals to habituate. Jelly cubes containing the analgesic (1 capsule per ice cube size jelly) was also given to the animals.

On the day of burn wound creation the rats were given an injection of 10 mg/kg Tramadol intramuscularly (IM) 15 minutes before wound creation. One hour post-wound creation another injection of Tramadol (10 mg/kg) was administered.

On day 2 post-wound creation the rats were divided into three groups. Three of the six rats received Morphine subcutaneously every 4 hours. Another group of three rats received Butorphanol subcutaneously every 4 hours. The control group, the remainder of three rats also received the Buterphanol analgesic regimen. See Table 4.2 for the summary of analgesics administered:

Table 4.2: Summary of analgesics administered

Group	Group 1 and Control group	Group 2
Day -1	Tramadol in drinking water (6 mg/ml) Tramadol in jelly cubes (50 mg per cube)	Tramadol in drinking water (6 mg/ml). Tramadol in jelly cubes (50 mg per cube)
Day 0	Tramadol in drinking water (6 mg/ml) Tramadol (10 mg/kg) administered Intramuscularly 15 min before wound creation 1 Hour post burn, Tramadol administered IM (10 mg/kg)	Tramadol in drinking water (6 mg/ml) Tramadol (10 mg/kg) administered IM 15 min before wound creation 1 Hour post burn, Tramadol administered IM (10 mg/kg)
Day 1	Tramadol administered IM (10 mg/kg)	Tramadol administered IM (10 mg/kg)
Day 2 - 7:	Butorphanol administered (2 mg/kg) SC every 4 hours	Morphine administered (10 mg/kg) SC every 4 hours
Summary:	Tramadol followed by Butorphanol	Tramadol followed by Morphine

In order to evaluate the welfare of the experimental animals and thereby the effectiveness of the analgesic regimen the following criteria were evaluated and documented (Table 4.3):

Table 4.3: Animal welfare score sheet

Protocol No: H20/08 J. Olivier				Animal no		
				Page no:		
Date						
Day						
Time						
General Clinical Signs:						
Inactive/Active						
Hunched posture						
Rough Coat / fur on end						
Red eye / nose discharge						
*Dehydration						
**Breathing						
Behavioural signs of pain:						
Back arch						
Belly press						
Vocalization						
Writhe						
Stagger						
Falling						
Weight H_2O Bottle						
Evidence of food eaten						
Jelly eaten						
Body weight (g)						
Body temperature °C						
Presence of feaces						
***Condition (4 – 1)						
OTHER NOTES:						
Signature / Initials						

Assess animals 4x per day post burn until end of the experiment.
*Dehydration – tent the skin: If >5% dehydration is clinically significant (the skin turgor will be lost)
<2 = moderate dehydration > 2 sec = severe dehydration.
**Breathing: R = Rapid; S = Shallow; L = Laboured; N = Normal
***Condition: 4 = Normal, 1 = Emaciated

4.2.5. Statistical analysis

The two analgesic treatment groups were compared using the student's t-test or unpaired t-test. Differences were considered statistically significant when the p-value was smaller than 0.05, resulting in a confidence interval of 95% (www.graphpad.com).

4.3. RESULTS

Since this part of the study is only concerned with establishing the burn wound model, in particular the analgesic regimen, it was not necessary to include the control group that did not receive the novel silk protein based wound dressing. Additionally, in this way all variables are kept to a minimum.

4.3.1 General clinical signs:

Activity:

The animals were observed every 6 hours post burn for the duration of the study. On the day of wound creation (day 0) all six rats were inactive, however, they were still alert. On day 1 post wound creation, all six rats were active and alert. All six animals were inactive during the second day post wound creation. Rat number 1, 5 and 6 were more active and alert in comparison to the other rats. Rat 2, 3 and 4 were less active, sleeping most of the time, however they were still alert.

Breathing Pattern:

During the first 3 days (day 0 – 2) of the study all animals showed a rapid breathing pattern on at least 1 observation interval per day. After day 3 their breathing patterns returned to normal.

Other clinical signs:

Hunched posture; rough coat / fur on end; red eye / nose discharge and dehydration were also observed for a minimum of 5 min every 6 hours for the duration of the study. During the first 3 days (day 0 – 2) of the study all animals

showed some discharge, this normalised after day 2. All other clinical signs were observed to be normal throughout the study.

4.3.2. Behavioural signs of pain:

General observations:

Back arch; belly press; vocalization; writhe; stagger and falling were also observed for a minimum of 5 min every 6 hours for the duration of the study. These behavioural signs of pain were observed to be normal when the animals were active and not asleep. It was also observed that the animals were sleeping in a curled up position.

Weight of water bottle and evidence of food eaten:

On day 0 and 1 it was evident that all the animals were eating and drinking. On day 2 it was observed that the animals stopped eating and weren't drinking their medicated water.

Weight change over time:

Weight was measured every day for the duration of the study. All the animals lost some weight and remained relatively stable during the first 2 days (day 0 – day 1) of the study. On day 2 it was observed that the animals were losing a significant amount of weight. From day 3 the animals gained a significant amount of weight and it was also observed that the net weight change over time, when considering the entire study, was positive for all six animals. See graph 4.1.

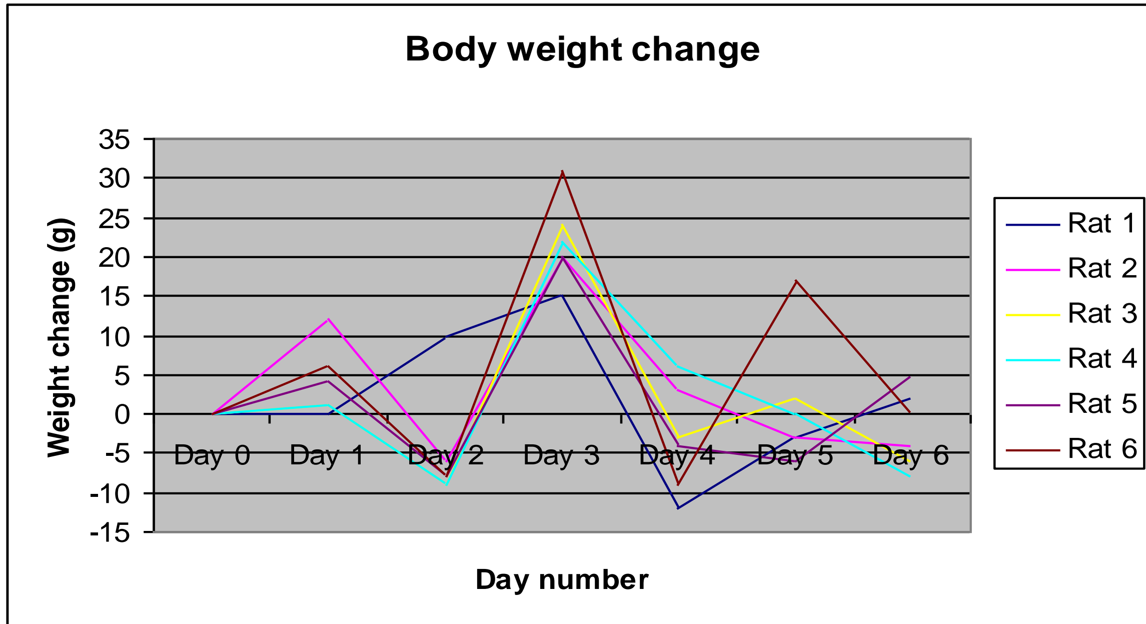
When comparing the two groups (with regards to the analgesic regimen), the group receiving the Butorphanol lost more weight in comparison to the group receiving the Morphine. See graph 4.2

Body temperature:

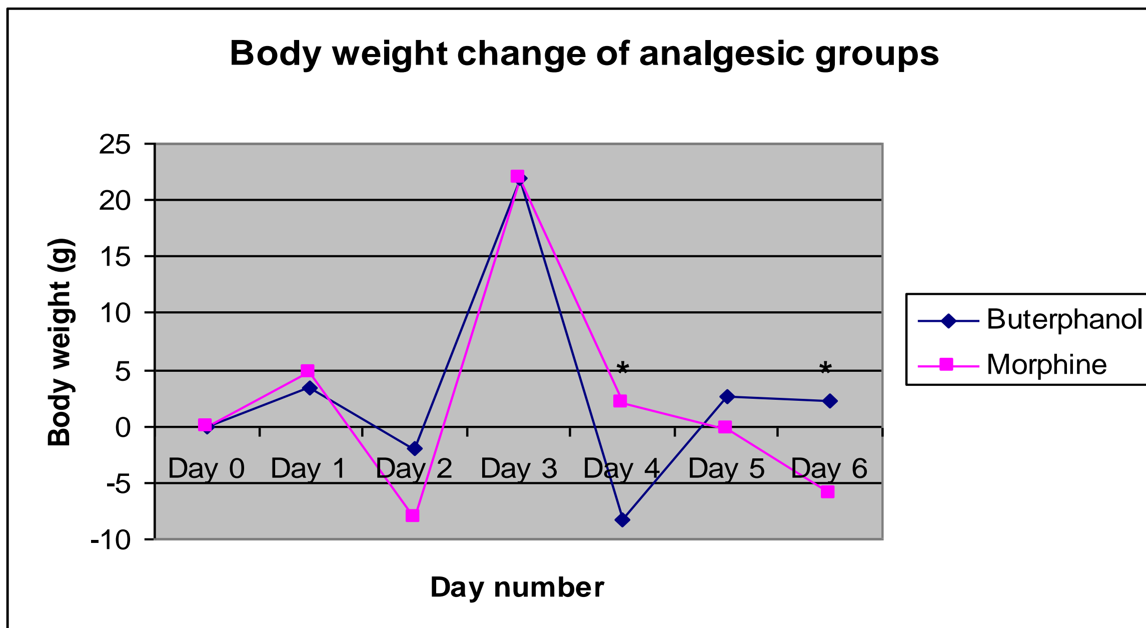
Body temperature was taken everyday for the duration of the study. The body temperatures of the experimental animals were slightly lower than normal when

measured under anaesthesia. The results did not indicate any extreme changes in body temperature. See graph 4.3.

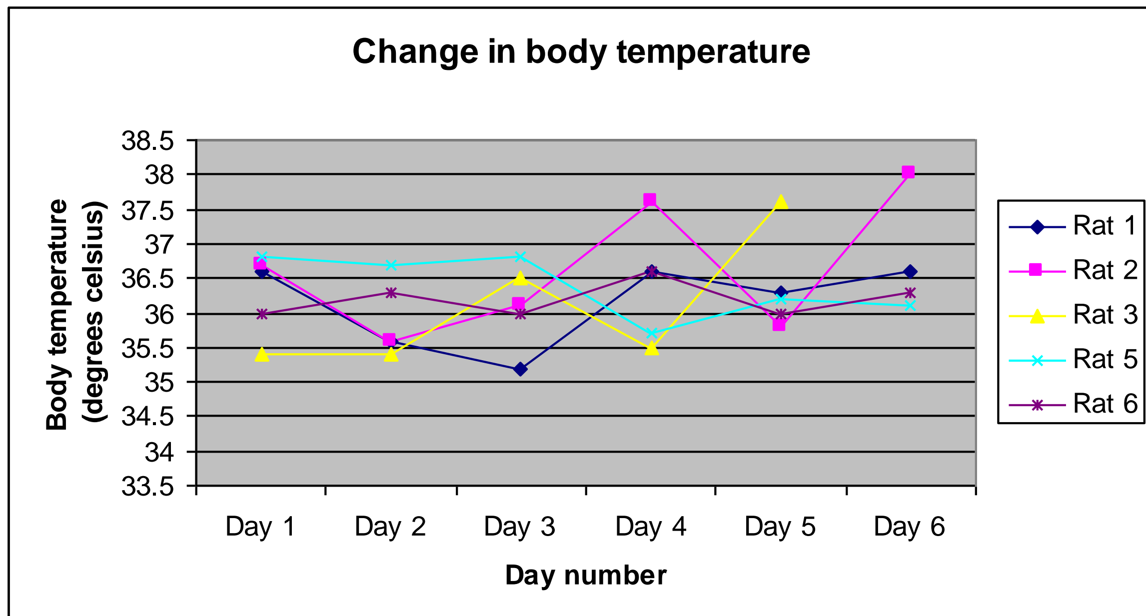
Graph 4.1: Body weight change over time, measured over 7 days.



Graph 4.2: Body weight change over time comparing the two analgesic groups measured over 7 days (* indicates statistical significant difference with $p < 0.05$).



Graph 4.3: Body temperature measured on a daily basis, measured over 7 days.



4.5. DISCUSSION

4.5.1 General clinical signs:

The animals were observed every 6 hours post burn for the duration of the study. On the day of wound creation (day 0) all six rats were inactive, but they were still alert. On day 1 post wound creation, all six rats were active and alert. However during the second day post wound creation all six animals were observed to be inactive. This change in activity from day 1 to day 2 might be considered an indication of pain (Roughan and Flecknell, 2003; Flecknell and Davies, 2004). This was one of the facts considered when making the decision to change the analgesic regimen.

The animals were then divided into two groups. Group 1 (Rat number 1, 5 and 6) received Butorphanol for the remainder of the study while group 2 (Rat 2, 3 and 4) received Morphine for the remainder of the study. The first group was observed to be more active and alert in comparison to the second group. The group receiving

Morphine as part of their analgesic regimen was less active than the group receiving Butorphanol, sleeping most of the time, however they were still alert. It may be concluded that the Morphine might have a slight sedative effect on the animals while Butorphanol is less sedative. This was expected since the literature suggests that Morphine has a sedative effect (Masica *et al*., 2007).

During the first 3 days (day 0 – 2) of the study all animals showed a rapid breathing pattern on at least 1 observation intervals per day. Rapid breathing patterns may also be an indication of pain (Roughan and Flecknell, 2003; Flecknell and Davies, 2004). This was also considered when deciding to change the analgesic regimen. After administration of Butorphanol or Morphine both groups' breathing patterns were observed to be normal throughout the remainder of the study.

During the first 3 days (day 0 – 2) of the study all animals showed some red eye or nose discharge. A reddish eye or nose discharge is known as an indication of stress (Flecknell and Davies, 2004). After day 2 neither group one nor group 2 showed any more signs of reddish discharge, possibly indicating the effectiveness of the new analgesic regimen. All other clinical signs were observed to be normal throughout the study for both groups.

4.5.2. Behavioural signs of pain:

Back arch; belly press; vocalization; writhe; stagger; and falling were observed for a minimum of 5 min every 6 hours for the duration of the study. These behavioural signs of pain were observed to be normal when the animals were active and not asleep. It may therefore be concluded that pain experienced was not severe (Roughan and Flecknell, 2003; Flecknell and Davies, 2004). It was also observed that the animals were sleeping in a curled up position, this may indicate that they were comfortable.

On day 0 and 1 it was evident that all the animals were eating and drinking, however on day 2 it was observed that the animals stopped eating and weren't

drinking their medicated water. This was confirmed by the body weight change recorded daily. The body weight of each individual rat was measured every day for the duration of the study. It became evident that all the animals' body weights remained relatively stable during the first two days of the study (day 0 and day 1). On day 3 it was observed that the animals were losing a significant amount of weight. This was considered when making the decision to change the analgesic regimen. After changing the analgesic regimen all the animals gained a significant amount of weight and it was observed that the net weight change when considering the entire study was positive for all six animals. See graph 4.1.

When comparing the two groups (with regards to the analgesic regimen), the group receiving the Butorphanol lost statistically significantly more weight in comparison to the group receiving the Morphine. This may be due to the sedative effect of Morphine. The animals receiving Butorphanol were also observed to be more active and may have lost weight due to a higher rate of physical activity (movement) during waking hours. See graph 4.2.

The body temperature of each individual rat was taken everyday for the duration of the study. The body temperatures of the experimental animals were slightly lower than normal when measured under anaesthesia. This was expected (Hall, 1978; Olsson and Hahn, 1996). The results didn't indicate any extreme changes in body temperature. See graph 4.3.

4.6. CONCLUSION

Tramadol administered *ad libitum* did not prove to be effective since the animals did not drink the medicated water as expected. It was therefore necessary to change the analgesic regimen to injectable analgesics.

Morphine and Butorphanol both proved to be effective. When comparing the two analgesic regimens the Butorphanol shows to be the better choice. The animals

receiving Butorphanol were observed to be more active and alert than the animals receiving Morphine. It is known that Morphine has a sedative effect and it may be due to this property.

Butorphanol and Morphine injected subcutaneously proves to be a good choice for a study running for a short period of time such as 7 days. Handling of the animals as well as the injections themselves showed to be somewhat traumatic to the animals. Both these medications need to be injected every 4 hours in order to be effective; the logistics concerned with this study would be difficult for a study running for a longer period of time.

Administering Butorphanol subcutaneously as part of the analgesic regimen in a burn wound study can be recommended for a short term study.

Chapter 5: Histological investigation of the novel silk protein based wound dressing's effect on wound healing: A macro- and microscopic evaluation

5.1. INTRODUCTION:

After injury of the skin, the resulting wound heals either by means of primary or secondary intention. Wounds that heal by secondary intention are those whose edges remain open. Alternatively, wounds that heal by primary intention are those that are closed early on by means of suture or stapling (Cohen *et al*., 1992). For the purposes of this study I will only focus on healing by means of secondary intention.

During healing by means of secondary intention the main mechanism is contraction, with the addition of epithelialization and connective tissue deposition. The actual biological mechanism of wound healing has been studied intensively and is an intricate and structured response that involves cellular and extracellular matrix components. The process is simplified by dividing it into four categories of normal healing: hemostasis, inflammation, proliferation and remodeling (Fonder *et al*., 2008; Cohen *et al*., 1992; Monaco and Lawrence 2003).

Once the injury occurs the epidermis and dermis are disrupted and the cutaneous vasculature is damaged. Blood cells spill unto the wound site and platelets come into contact with exposed collagen and other tissue fragments, this triggers the first healing processes to establish hemostasis. The platelets aggregate and degranulate. A thrombus forms via the deposition and polymerization of fibrin as well as the continuation of platelet aggregation. Simultaneously reactive vasoconstriction of the damaged blood vessels occurs. This, in conjunction with the formation of the thrombus, leads to hemostasis (Cohen *et al*., 1992; Monaco and Lawrence 2003). In addition to their role in hemostasis blood platelets also

influence the cellular response within the wound by releasing platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). PDGF is a chemoattractant for smooth muscle cells and fibroblasts. TGF-β has a similar chemoattractive effect on inflammatory cells and fibroblasts (Duncan *et al*., 1999; Cohen *et al*., 1992; Monaco and Lawrence 2003). The chemoattraction of inflammatory cells are part of the first steps to move into the next category of normal healing, namely inflammation.

Acute inflammation follows as a result of vasodilation, increased capillary permeability and the infiltration of neutrophils into the wound site (Cohen *et al*., 1992). Neutrophils control local bacterial contamination and aid in the debridement of necrotic and devitalized tissue. After 24 hours infiltration recedes as monocytes start to infiltrate the wound site. Monocytes are converted to macrophages that continue to eliminate bacteria and necrotic cells (Fonder *et al*., 2008; Cohen *et al*., 1992). Monocytes also secrete growth factors which stimulates fibroblast proliferation and collagen synthesis. Additionally macrophages secrete cytokines such as IL-1 and TNF-α. During the inflammatory phase the wound matrix also changes. Vascular permeability increases, transudation of plasma components follows and compliment antibodies and other plasma components enter the wound. Fibrin is replaced by glycosaminoglycans and proteoglycans during transudation allowing the wound to enter the next stage of healing (Cohen *et al*., 1992; Monaco and Lawrence 2003).

The next stage is the proliferative stage and can be characterized by the production of collagen, granulation tissue formation and neovascularization (Fonder *et al*., 2008). During the proliferation phase, in full thickness wounds, the damaged dermis heals primarily through connective tissue deposition, especially collagen crosslinking. The most important cell in this process is the fibroblast. Fibroblast infiltration and proliferation peaks at about 3 to 4 days after injury. This is mainly due to the secretion of growth factors that stimulate the migration and activity of these cells during the previous phases of wound healing.

Simultaneously, proliferation of endothelial cells also occur, initiating neovascularization. Cellular migration at this stage is guided by the matrix within the wound. Cells migrate across anatomical tissue planes and planes aligned according to the tension across the wound. The reason why fibroblast infiltration is crucial for normal wound healing is because these cells are primarily responsible for collagen synthesis. The deposition of collagen into the wound is concurrent with the degradation of the proteoglycans, which initially made up the wound matrix. Collagen type III is initially deposited in the healing wound and later, during the next stage, replaced by collagen type I, the predominant collagen of skin.

Remodeling is the stage of the healing wound that takes the longest. During this period the neovascularisation of early granulation tissue recedes as repair continues. The main processes in this wound healing stage is remodeling of collagen (Cohen *et al*., 1992). In virtually all tissues, including wounds, there is a balance between collagenolytic activity and collagen synthesis resulting in a net deposition of collagen. Therefore collagen production and degradation is an ongoing process (Monaco and Lawrence 2003; Cohen *et al*., 1992). Even though there is a reduction in collagen synthesis during remodeling, the tensile strength still increases. The increase in tensile strength is a result of structural modification of the newly deposited collagen (Cohen *et al*., 1992).

Collagen deposited after 5 days of healing consist mainly of fine unorganized fibrils. Over time the fibril diameter increases, fascicles form and the fibers become more compact. Cross-linking of collagen fibrils is largely responsible for structural modification of newly deposited collagen resulting in increased strength. Cross-linking of collagen is achieved by the formation of covalent bonds. This reaction is mediated by the enzyme lysyl oxidase (Redden and Doolin, 2003; Cohen *et al*., 1992). However, the newly formed skin never achieves the same tensile strength as normal skin and the collagen never achieves the normal structure of dermal collagen (Cohen *et al*., 1992).

It is well known that collagen formation, cross-linking and remodeling is a crucial part of normal wound healing. It would therefore be beneficial if a wound dressing was able to improve this function of healing. Sericin has been documented to improve epithelialization and collagen deposition in a Sprague-Dawley rat model (Aramwit and Sangcakul, 2007). Fibroin has also been documented to have a promotive effect on collagen production and deposition (Sugihara *et al.* 2000). The novel silk protein based wound dressing also contains copper ions. Literature indicates that copper is an important trace element in many physiological functions including its role as a co-factor (Gupte and Mumper, 2009). It activates VEGF, bFGF, TNF-α and IL-1. Through these factors copper stimulates angiogenesis and endothelial proliferation and migration (Nasulewicz *et al.*, 2004).

In the current study the Wistar rat model was used to evaluate the histological effects of the novel silk protein based wound dressing. The first question that arises is whether the silk protein based wound dressing will positively effect granulation and epithelailization. The second question that arises is whether the novel silk protein based wound dressing will improve the collagen deposition.

5.2. MATERIALS AND METHODS:

5.2.1. Implementing the burn wound model:

Nine female Wistar albino rats weighing between 200 – 250g, maintained at the Uiniversity of Pretoria Biomedical Research Centre were used in this study. The rats were kept in a room with a 12h light and dark cycle and a room temperature constant at 22 °C. The rats were fed a standard rat diet, water *ad libitum* and were housed in individual polycarbonate cages. The rats were fasted for 12h before thermal injury; but they had free access to water. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee.

This part of the study was concerned with the effect of a novel silk protein based wound dressing on wound healing. Six rats received treatment with a novel silk protein based wound dressing. The remaining three rats were not treated with the novel silk protein based wound dressing. This was the control group.

5.2.2. Thermal injury

On the first day (day 0), the rats were anaesthetized with Isoflurane and directly afterwards the analgesic, Tramadol (10 mg/kg), was injected intramuscularly. After injection of the analgesic, 15 minutes was allowed before wound creation in order to allow the analgesic to take effect. The dorsum of each rat was shaved and then exposed to a 1 x 1 cm brass block for 10 seconds (Gottrup *et al.*, 2000; Dorsett-Martin and Wysocki, 2008). This brass block was heated up to 95 °C using a hot water bath. Using gravity only, the brass block was rested on the dorsum of the rats, which resulted in partial thickness skin burns (Mogford and Mustoe, 2001; Dorsett-Martin and Wysocki, 2008). A physiological saline solution was then administrated intraperitoneally (25 mg/kg) in order to prevent dehydration of the animal.

5.2.3 Treatment

Immediately after thermal injury all wounds were dressed with a gauze primary dressing and Opsite® as the secondary dressing. In order to keep the rats from interfering with the healing process the secondary dressing was further covered with a third dressing of bandage and fastened with Elastoplast® around the edges.

On day 3 post-wound creation the rats were divided into two groups. During the dressing changes one group of three rats received the normal dressing changes composed of gauze and Opsite®. This was the control group. Another group of six rats received the normal dressing change composed of gauze and Opsite®, but their wounds were treated with an additional novel silk protein based primary wound dressing.

5.2.4 Preparation of the tissue samples

On the day of termination (7 days post-burn) one sample of the wound edge from each rat, across the area of visible granulation tissue formation, was taken by means of a punch biopsy. This sample was immediately fixed in 10% buffer formalin solution. The samples were then embedded in paraffin wax. This was done by first dehydrating the samples in serial dilutions of 70%, 90% and 100 % ethanol solutions for 2 hours each. The samples were then infiltrated with wax by first submerging them in xylene for 2 hours and then in a 30%, 70% and finally 100% wax-xylene solution for 1 hour. Each sample was then embedded in a separate paraffin wax block and 5µm sections were made. These sections were then mounted and stained. After mounting the tissue samples every other section is stained with either Hematoxylin Eosin stain or Massons' Trichrome stain. The histological tissue samples were then viewed under a 40 x magnifications lens with a light microscope.

5.2.5 Hematoxylin and Eosin staining

Hematoxylin is a nuclear stain that stains the nuclei of the cell blue to black. The counterstain, Eosin stains the cytoplasm and connective tissue a pinkish colour. The mounted tissue sections were deparaffinize in 2 changes of xylene for 2 minutes each and then rinse in 2 changes of absolute ethanol for 2 minutes each. The sections were then rinsed in serial dilutions of 90%, 70% ethanol and then distilled water for 60 seconds each. After depariffinization the sections were stained in Hematoxylin solution for 5 to 10 minutes, washed in tap water for 10 minutes and then rinsed with distilled water. The sections were then counter-stained with Eosin for 3 minutes and again rinsed in distilled water. After staining the sections, they were rinsed in serial dilutions of ethanol, firstly 70%, then 90% and finally in 2 changes of absolute ethanol for 2 minutes each. The sections were then rinsed in 2 changes of xylene for 2 minutes each and mounted with a cover slip and mounting medium (Lillie, 1977; Humason, 1979 and Hayat, 1993).

5.2.6 Masson's Trichrome staining

Trichrome stains are used to differentiate between collagen and smooth muscle and to identify increases in collagenous tissue. In this study Masson's Trichrome is used to identify an increase in collagen deposition in healing tissue. This staining method stains the nuclei black and collagen and mucus blue. The cytoplasm, keratin and muscle fibers are stained red.

The mounted tissue sections were deparaffinize in 2 changes of xylene for 2 minutes each and then rinsed in 2 changes of absolute ethanol for 2 minutes each. The sections were then rinsed in serial dilutions of 90%, 70% ethanol and then distilled water for 60 seconds each. After deparaffinization and hydration the sections were mordant in Bouin's solution for 1 hour at 56°C. The sections were then left to cool and rinsed in tap water until the yellow colour disappeared. Each section was then stained with Weigert's hematoxylin for 10 minutes and again washed in tap water for 10 minutes and rinsed in distilled water. The sections were then stained in Beibrich scarlet-acid fuchsin for 2 minutes and placed in phosphomolybdic-phosphotungstic acid solution for 15 minutes. The sections were then stained with aniline blue solution for 5 minutes and rinsed in distilled water. After staining with aniline blue each section was placed in a 1% acetic acid solution for 5 minutes, dehydrated with 2 changes of 95% ethanol and cleared with three changes of xylene. The sections were then mounted with a cover slip and synthetic resin (Lillie, 1977 and Humason, 1979).

5.3. RESULTS

Figure 5.1 shows the wounds of the treatment group compared with that of the control group. Photograph 1a shows the wound of a rat from the treatment group 7 days after thermal injury. Photograph 1b shows the wound of a rat from the control group 7 days after thermal injury. In comparison red granulation tissue is clearly visible around the edges of the treatment group wound at day 7, while that of the control group shows no granulation tissue.

Figure 5.2 shows a section of normal rat skin, taken from the same site as the burn, stained by H & E. The micrograph shows the different skin layers. The epidermis **E** consists of the *stratum corneum* **C**, the *stratum granulosum* **G**, *stratum spinosum* **S** and the *stratum basale* **B**. The dermis **D**, is composed of the papillary dermis **P** and the reticular dermis **R** (Coetzee *et al*., 2003).

Figure 5.3 shows a section of normal rat skin, taken from the same site as the burn, stained by H & E. The micrograph shows the panniculus carnosus muscle **M**, with visible striations **MS**. One significant difference between rat skin and human skin is that a rat has a subcutaneous panniculus carnosus muscle **M** as seen in this micrograph (Gottrup *et al*., 2000; Dorsett-Martin and Wysocki, 2008). The micrograph also shows the reticular dermis **R** of the dermis layer of skin.

Figure 5.4 shows a section of normal rat skin, taken from the same site as the burn, stained by Massons' trichrome. The micrograph shows the collagen fibers **Col** of the dermis. The dermis is composed of the papillary dermis **P** and reticular dermis **R**. The reticular dermis contains coarser, thicker collagen fibers, **Col**, than the papillary dermis.

Figures 5.5 and 5.6 both show sections of rat skin, taken from the burn site of different groups at day 7 post-burn. Figure 5.5 shows the histology of the burn site from the treatment group while figure 5.6 shows the histology of the burn site from the control group. The sections were stained by H & E. The treatment group (figure 5.5) shows epithelialization **EP** and the underlying dermal tissue **DT.** The control group (figure 5.6) shows no evidence of re-epithelialization. No difference in the dermal tissue between the treatment group and the control group could be found.

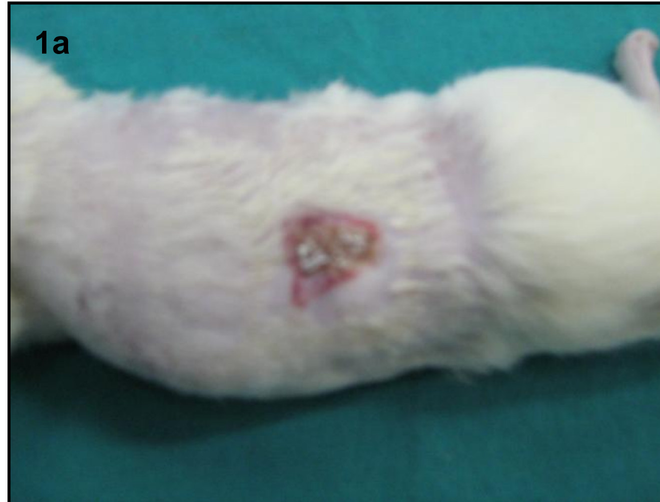
Figures 5.7 and 5.8 both show sections of rat skin, taken from the burn site of different groups at day 7 post-burn. Figure 5.7 shows the histology of the burn site from the treatment group while figure 5.8 shows the histology of the burn site from

the control group. The sections were stained by H & E. The treatment group (figure 5.7) shows newly formed blood vessels **BV** in the reticular dermis **R.** This indicates granulation tissue formation. The control group (figure 5.8) shows no evidence of granulation tissue formation.

Figures 5.9a and 5.9b show sections of rat skin, taken from the burn site of the treatment group at day 7 post-burn, stained by Masson's trichrome. The micrograph of figure 5.9a was taken using a 40x magnification lens while the micrograph of figure 5.9b was taken using a 20x magnification lens. In both figure 5.9a and 5.9b evidence of epithelialization **EP** is visible. Figure 5.9b shows epithelilization **EP** of the wound surface by epithelial cell migration from the follicles **F**. No difference in the dermal tissue between the treatment group and the control group could be found.

Figures 5.10, 5.11a and 5.11b show sections of rat skin, taken from the burn site of different groups at day 7 post-burn. Figure 5.10 shows the histology of the burn site from the treatment group while figures 5.11a and 5.11b shows the histology of the burn site from the control group. The sections were stained by Masson's trichrome. The treatment group (figure 5.10) shows newly formed blood vessels **BV** in the reticular dermis **R.** This indicates granulation tissue formation. The control group (figures 5.11a and 5.11b) shows no evidence of granulation tissue formation. However, it does show white blood cell **WBC** infiltration.

Figure 5.1: Photographs showing the burn wounds of the treatment group 7 days after thermal injury (1a) compared to the control group 7 days after thermal injury (1b).



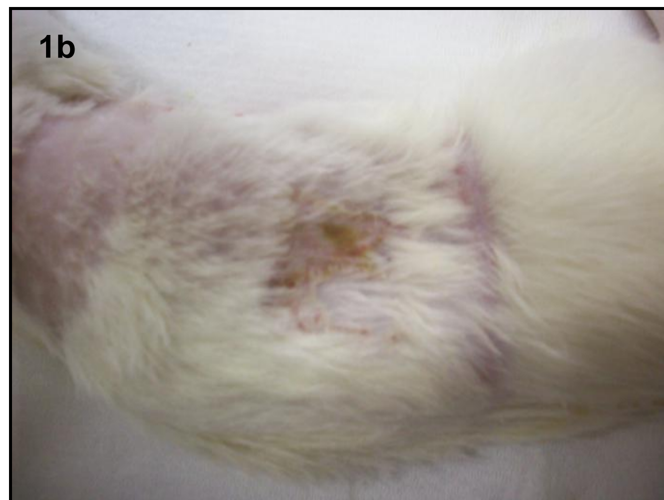


Figure 5.2: Histology of normal rat skin, taken from the same site as the burn, stained by H & E. The micrograph shows the different skin layers. The epidermis **E** consists of the *stratum corneum* **C**, the *stratum granulosum* **G**, *stratum spinosum* **S** and the *stratum basale* **B**. The dermis **D**, is composed of the papillary dermis **P** and the reticular dermis **R**. Scale = 20μm

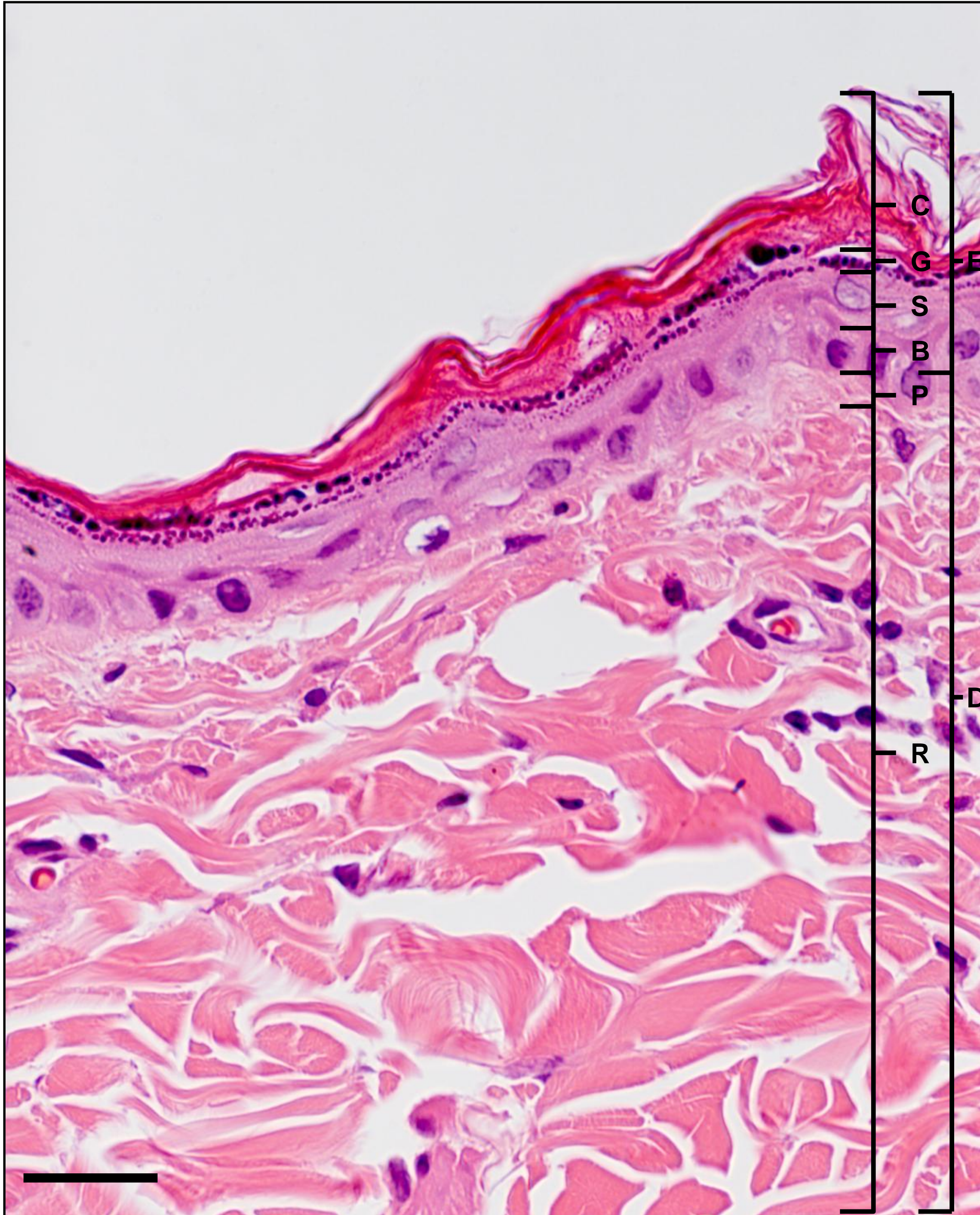


Figure 5.3: Histology of normal rat skin, taken from the same site as the burn, stained by H & E. The micrograph shows the panniculus carnosus muscle **M**, with striations **MS**; as well as the reticular dermis **R** of the dermis layer of skin.
Scale = 40μm

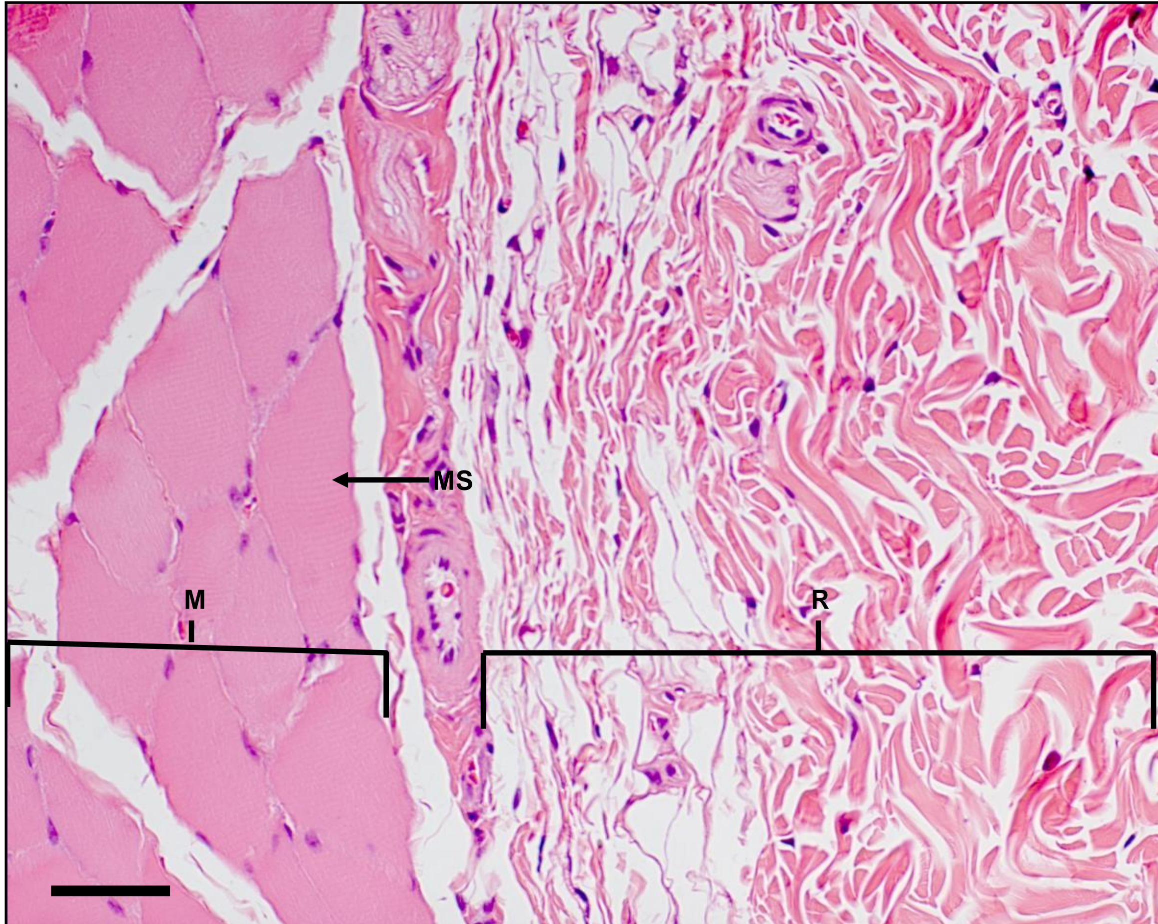


Figure 5.4: Histology of normal rat skin, taken from the same site as the burn, stained by Massons' trichrome. The micrograph shows the collagen layers: papillary dermis **P** and reticular dermis **R**. The reticular dermis contains coarser, thicker collagen fibers, **Col**, than the papillary dermis. Scale = 20μm

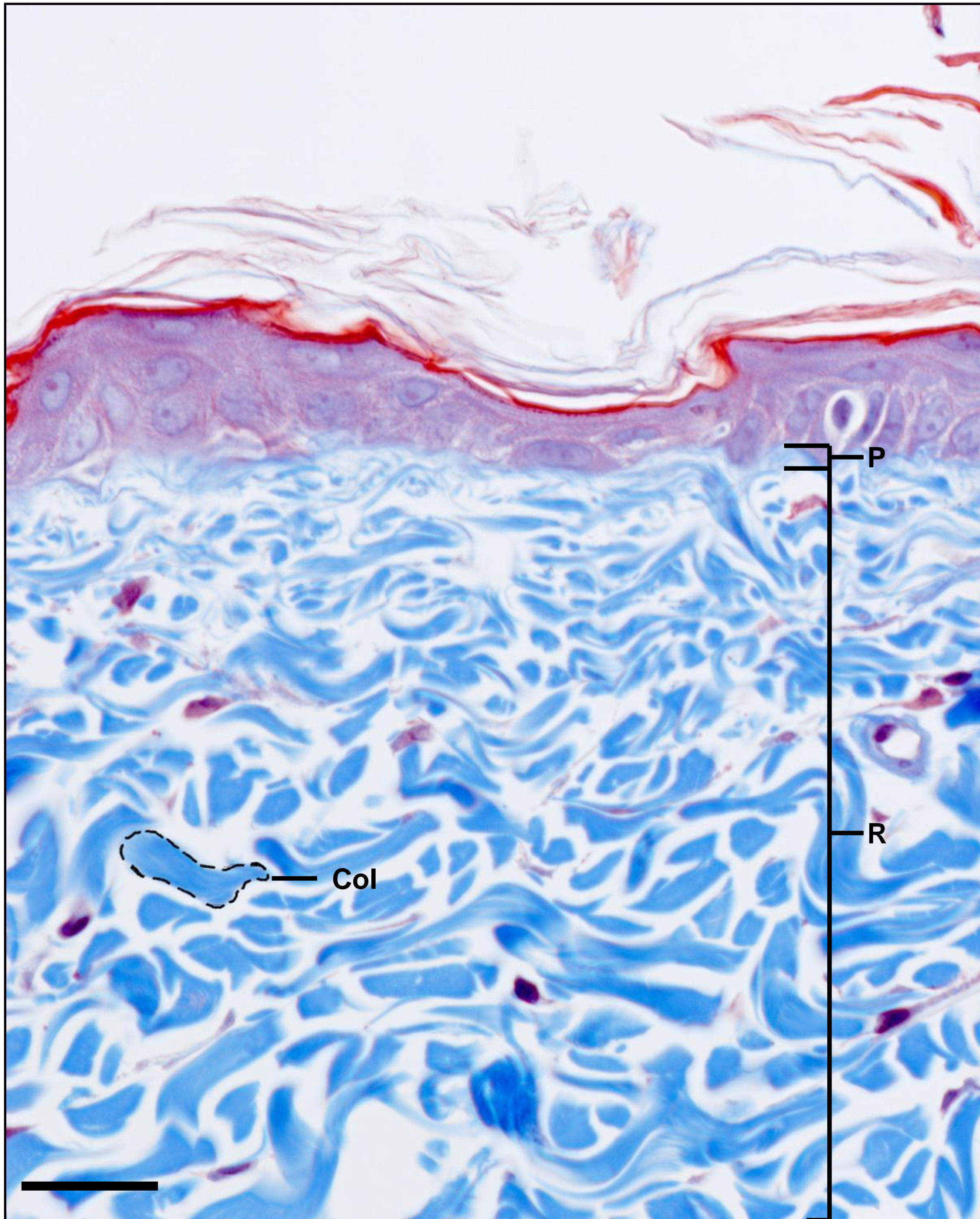


Figure 5.5: Histology of rat skin, taken from the burn site of the treatment group at day 7 post-burn, stained by H & E. The micrograph shows the wound surface **WS** showing epithelialization **EP** and the underlying dermal tissue **DT.**
Scale = 20μm.

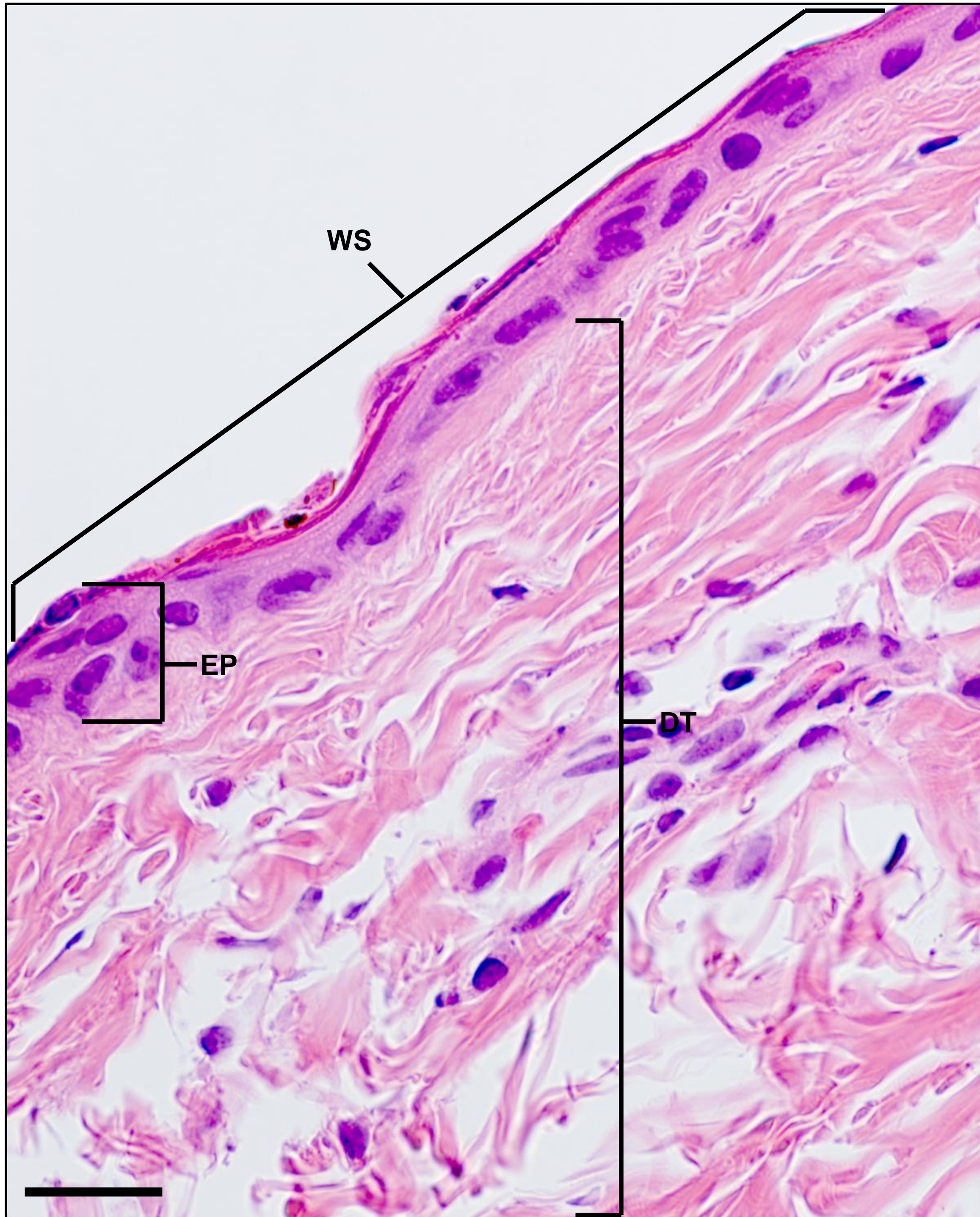


Figure 5.6: Histology of rat skin, taken from the burn site of the control group at day 7 post-burn, stained by H & E. The micrograph shows no epithelialization on the wound surface **WS** and the underlying dermal tissue **DT.** The underlying dermal tissue contains white blood cell infiltration **WBC**. Scale = 20µm.

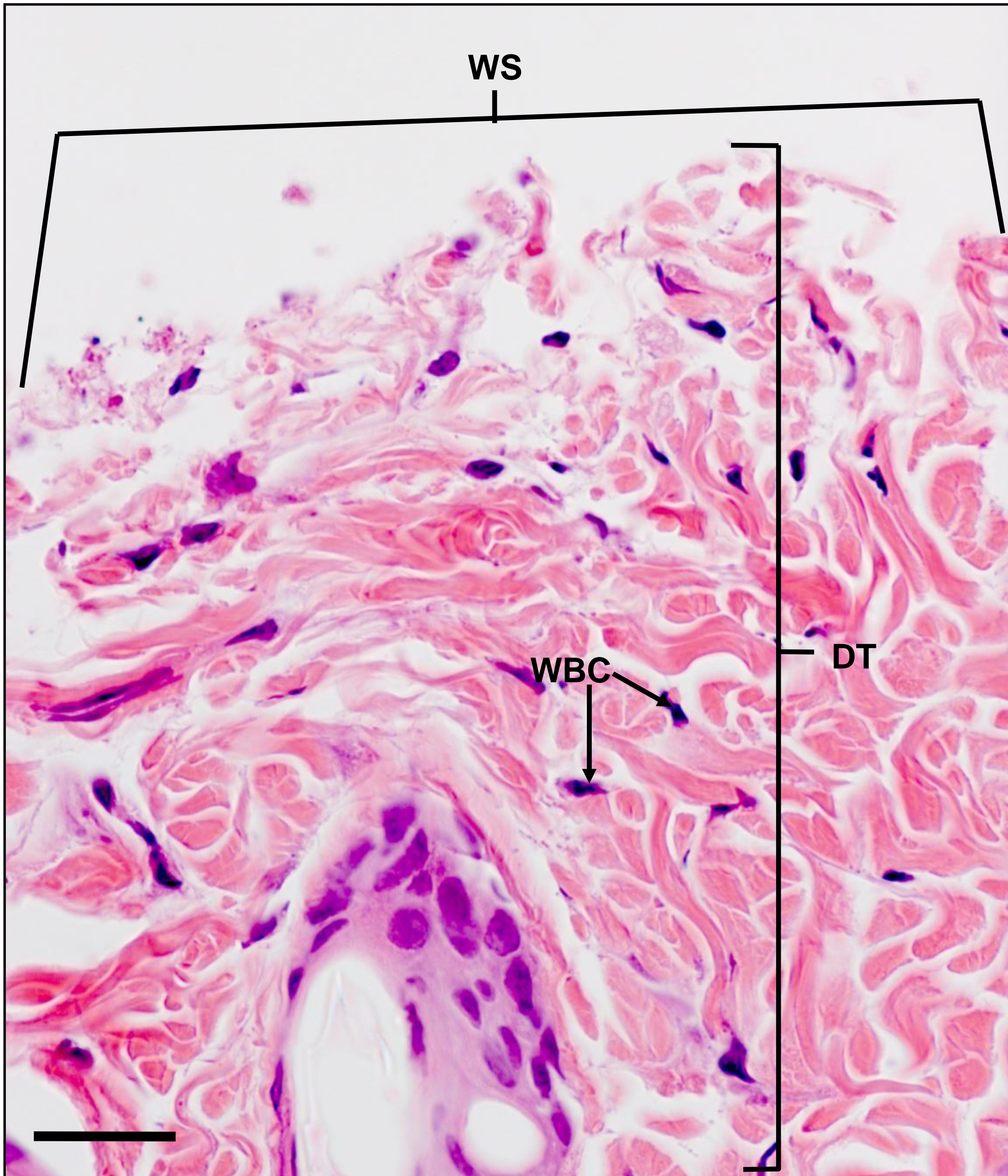


Figure 5.7: Histology of rat skin, taken from the burn site of the treatment group at day 7 post-burn, stained by H & E. The micrograph shows newly formed blood vessels **BV** in the reticular dermis **R** of the dermis indicative of granulation tissue formation**.** Scale = 20μm

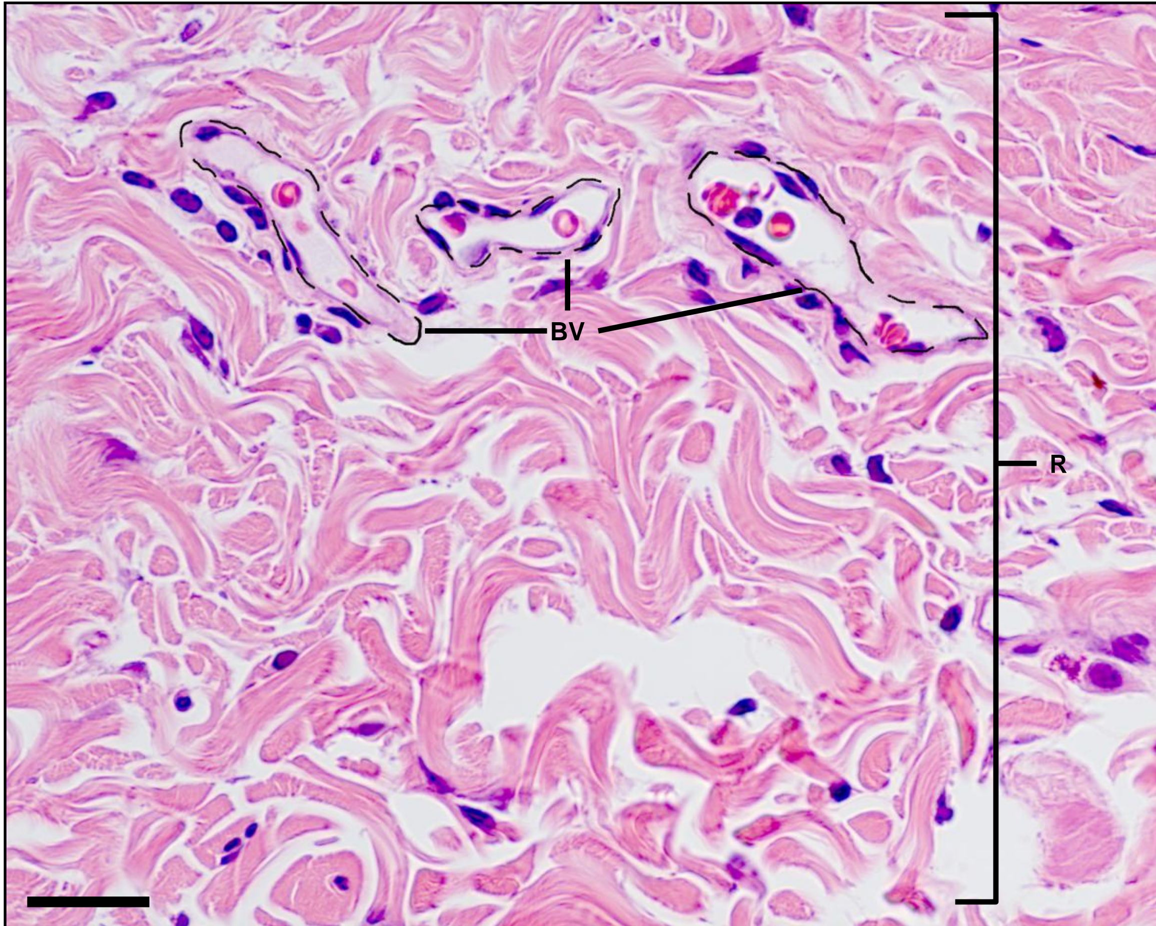


Figure 5.8: Histology of rat skin, taken from the burn site of the control group at day 7 post-burn, stained by H & E. The micrograph shows no newly formed blood vessels in the reticular dermis **R** of the dermis. The underlying dermal tissue **DT** contains white blood cell infiltration **WBC**. The wound surface is labeled **WS**.
Scale = 20μm.

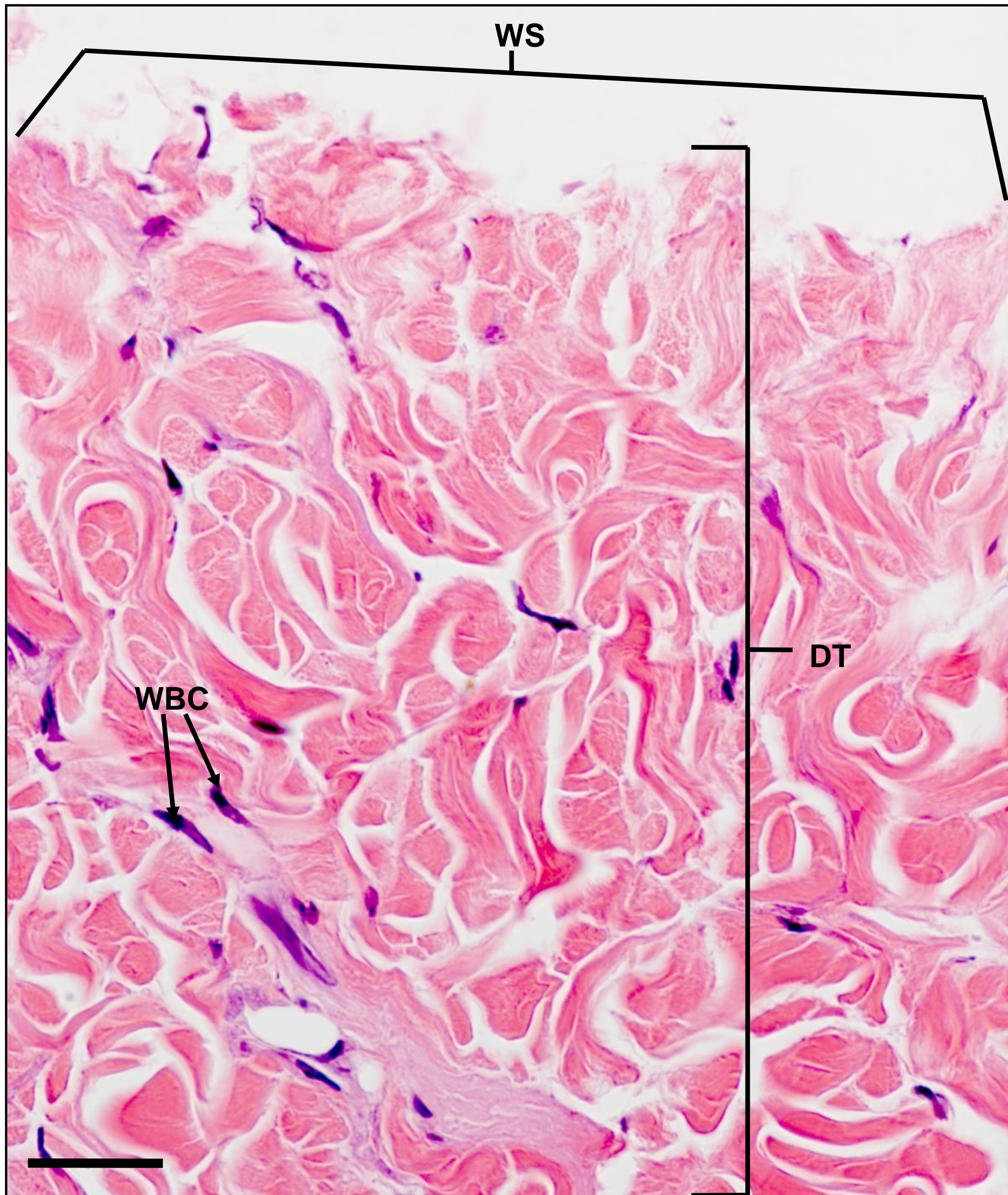


Figure 5.9a: Histology of rat skin, taken from the burn site of the treatment group at day 7 post-burn, stained by Masson's trichrome. The micrograph shows epithelialization **EP** on the wound surface **WS** and the underlying dermal tissue **DT.** Scale = 20µm

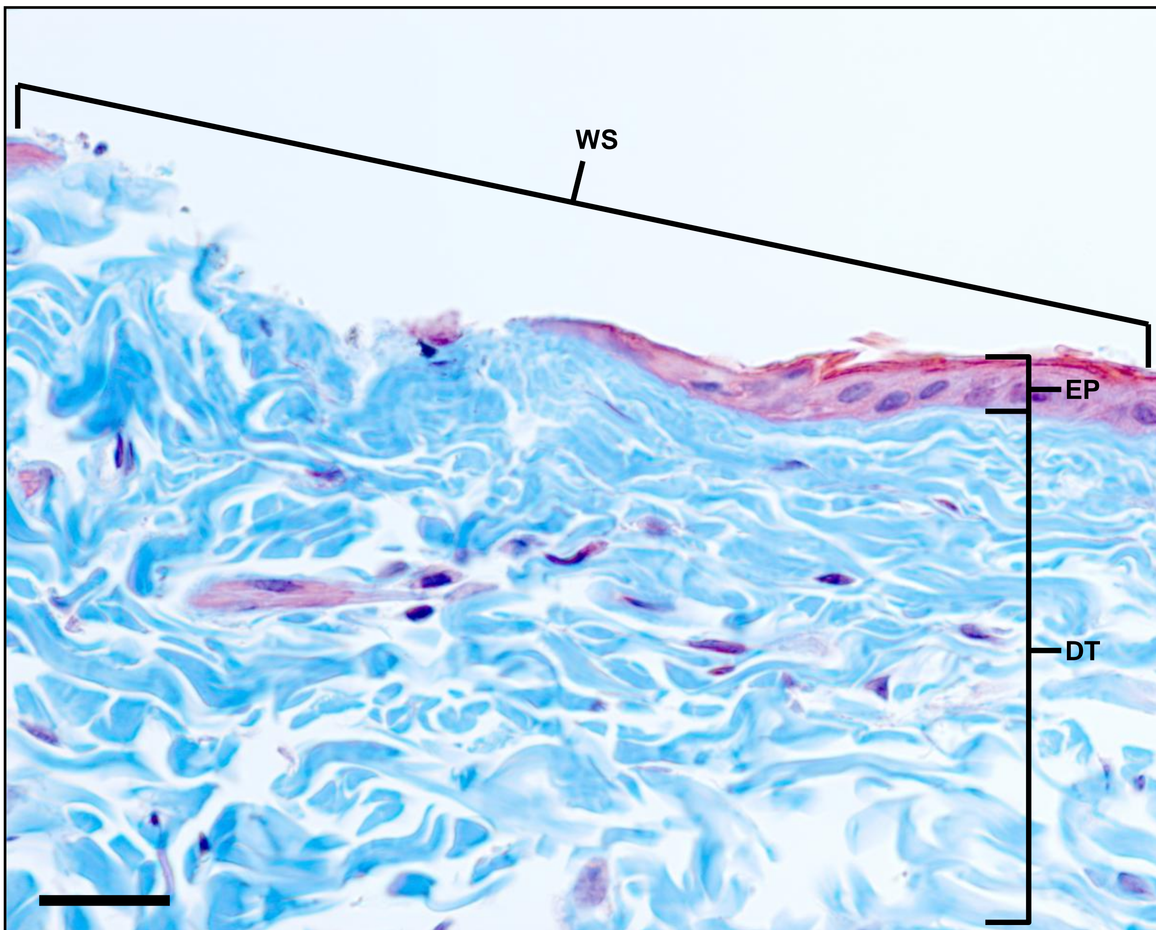


Figure 5.9b: Histology of rat skin, taken from the burn site of the treatment group at day 7 post-burn, stained by Masson's trichrome. The micrograph shows re-epithelialization **EP** on the wound surface **WS** from the hair follicles **F**. The micrograph also shows an epithelial cell **EC**. Sale = 40μm

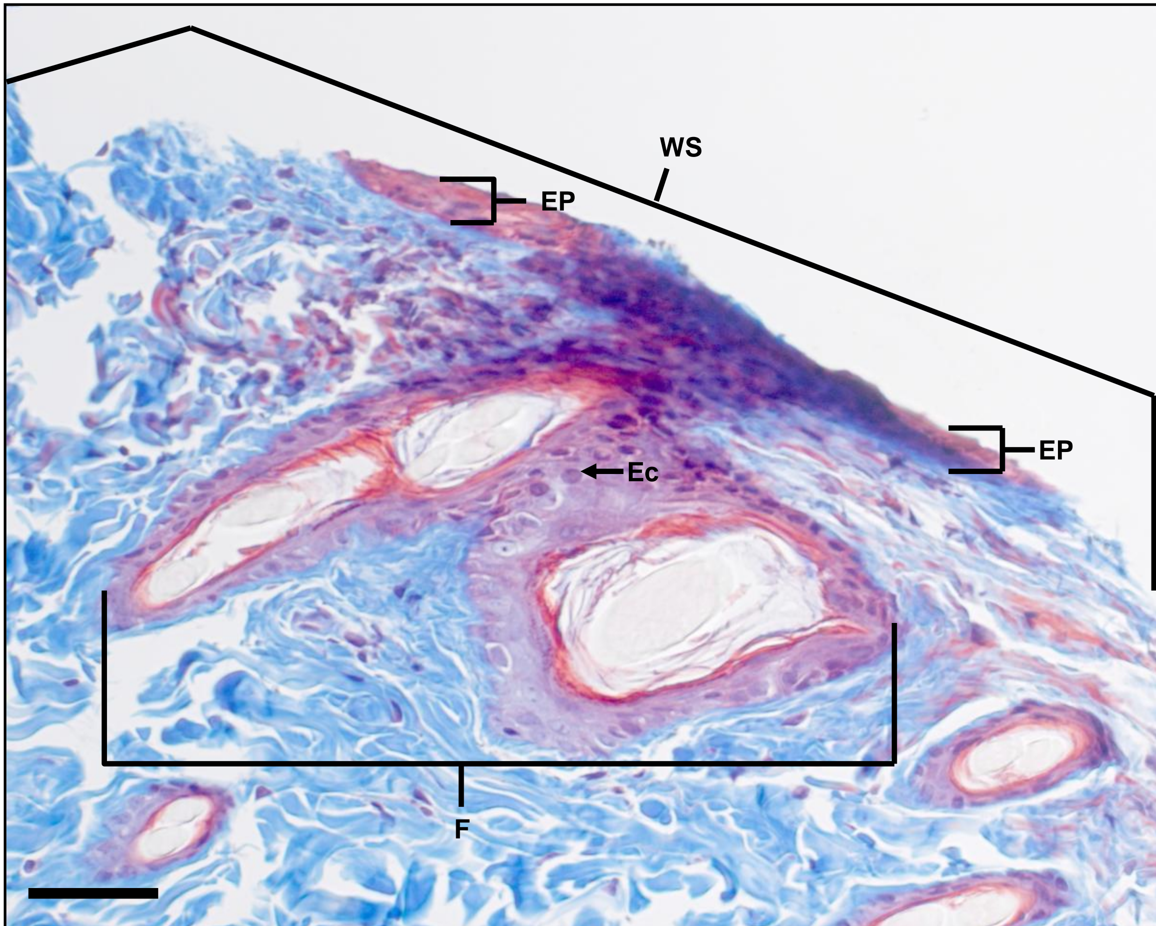


Figure 5.10: Histology of rat skin, taken from the burn site of the treatment group at day 7 post-burn, stained by Masson's trichrome. The micrograph shows newly formed blood vessels **BV** in the reticular dermis **R** of the dermis indicative of granulation tissue formation. Scale = 20 µ

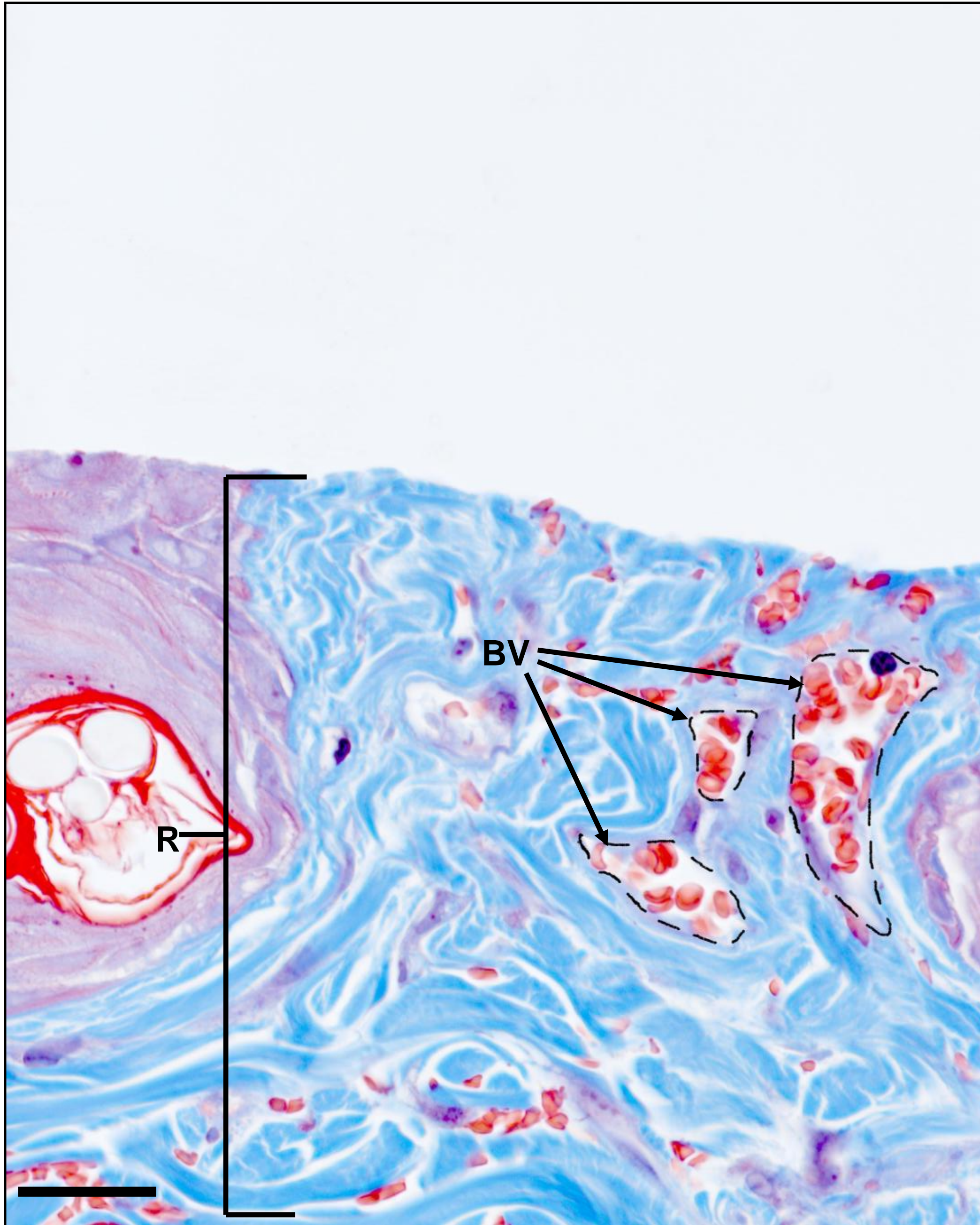


Figure 5.11a: Histology of rat skin, taken from the burn site of the control group at day 7 post-burn, stained by Masson's trichrome. The micrograph showings the dermal tissue **DT** with white blood cell **WBC** infiltration. The wound surface is labeled **WS**. No epithelialization or granulation tissue is present. Scale = 20μm

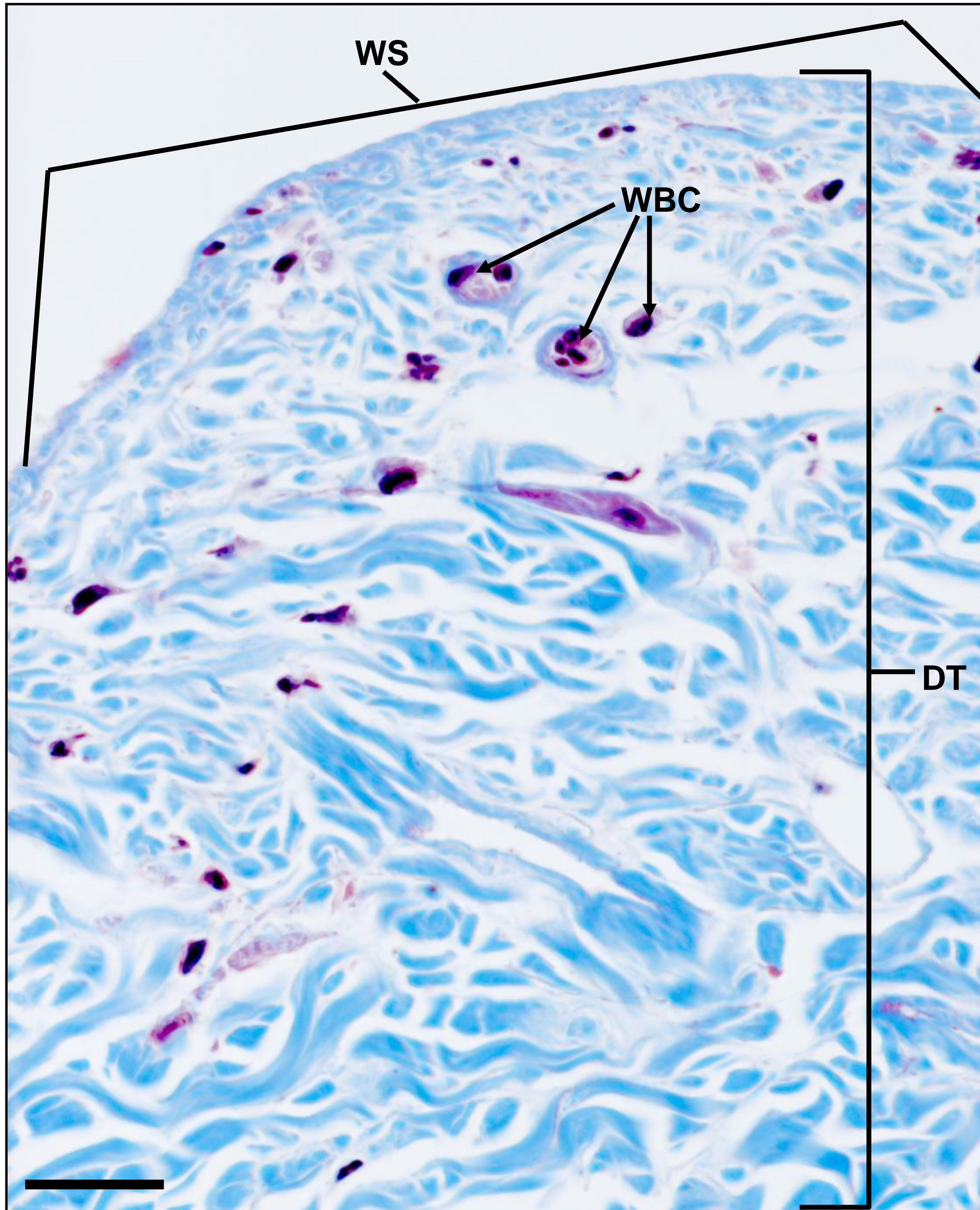
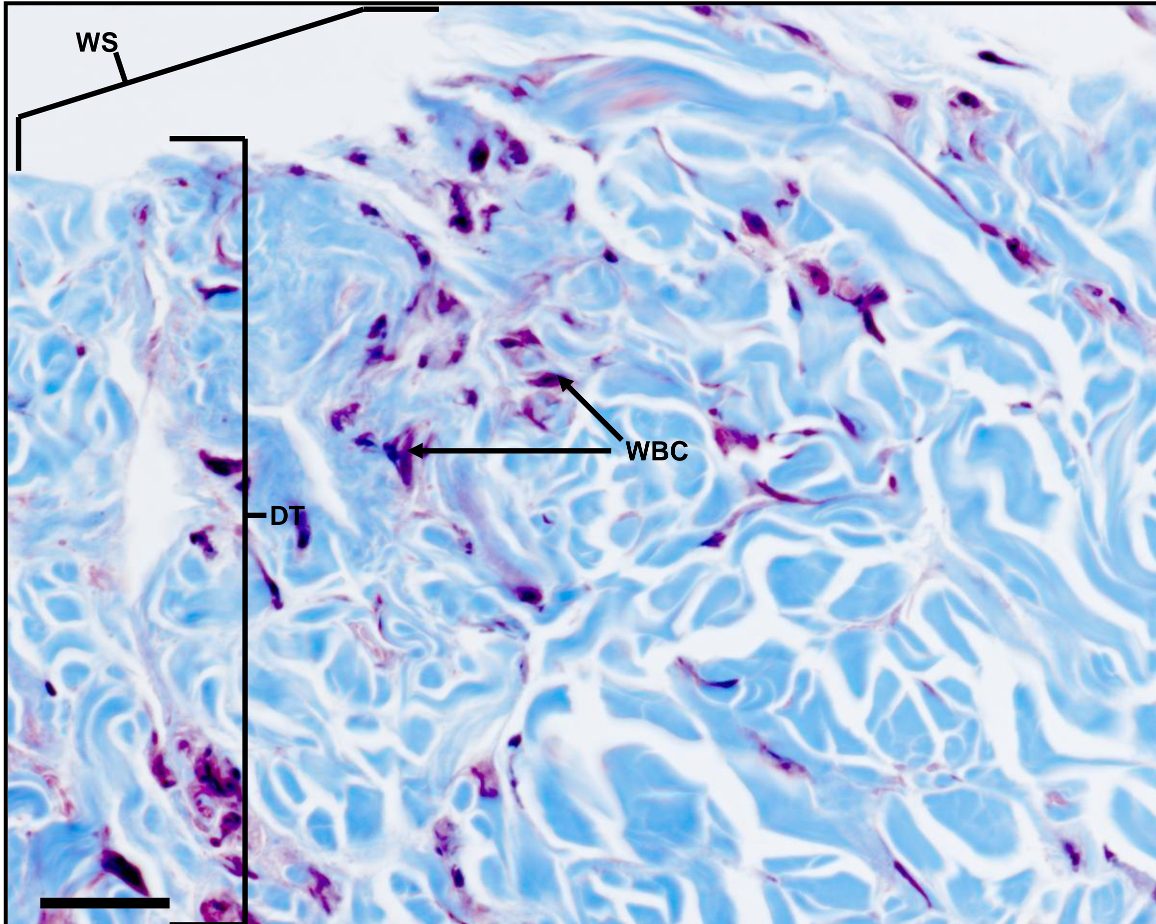


Figure 5.11b: Histology of rat skin, taken from the burn site of the control group at day 7 post-burn, stained by Masson's trichrome. The micrograph shows the dermal tissue **DT** with white blood cell **WBC** infiltration. The wound surface is indicated by **WS**. No evidence of epithelialization or granulation tissue is present. Scale = 20μm



5.4. DISCUSSION

Fibroblast infiltration and proliferation peaks at about 3 to 4 days after injury. Simultaneously, proliferation of endothelial cells also occur, initiating neovascularization (Cohen *et al*., 1992).These occurrences initiate the formation of granulation tissue containing blood vessels, and migration of epithelial cells. Granulation tissue formation and epithelialization is a characteristic of the proliferative phase of wound healing (Fonder *et al*., 2008). The proliferative phase starts 3 to 4 days after tissue injury (Cohen *et al*., 1992), for this reason the novel silk protein based wound dressing was applied 3 days after thermal injury on the burn wounds of the treatment group. Similar animal studies have been done and are documented in the literature.

Aramwit and Sangcakul (2007) evaluated the effect of sericin cream on wound healing. They tested its effect on 9 Sprague-Dawley rats by creating a 1.5 x 1.5 cm excision on the dorsum of each rat. They found that the inflammatory reaction of the wounds treated with the sericin cream was smaller than the control group throughout the study. During the first few days there was inflammation in both groups, however, this improved as of the tenth day after operation. Wound size was also measured throughout the study and expressed as a percentage of the initial wound size. The wound size was recorded on the day of operation as well as days 5, 10 and 15. Their results showed a statistically significant difference in the percentage of wound size reduction between the sericin treated group and the control group. The sericin treated group showed a complete reduction in wound size (100%) as compared to 91.7% (SD ±10.14) of the control group after 15 days. More than 70% of the wounds treated with sericin healed completely after 10 days of treatment

Aramwit and Sangcakul (2007) also evaluated the histology of the wounds by Hematoxylin Eosin staining and Masson's Trichrome staining. They found that the group of rats treated with sericin showed better epithelialization and collagen

deposition. The control group also showed some ulcers being present and they observed an uneven epidermis near the ulcer. The newly formed epidermis of the sericin treated group was even, with dense collagen. They further concluded from their results that sericin has wound healing properties without causing allergic reactions (Aramwit and Sangcakul, 2007).

A similar study was reported on by Sugihara and co-workers (2000). They studied the effect of a fibroin film wound dressing on full thickness skin wounds in mice. They created 1.5 x 0.9 cm dermatotomies on the dorsal wall of each mouse. They found that the area of the wound treated with the fibroin film dressing was reduced to 10% 14 days after operation. These areas were covered with regenerated epidermis 21 days after operation. The control group showed less recovery within the same time. The control group was covered with a conventional hydrocolloid dressing and showed less epidermal regeneration after 14 days. Furthermore only partial epidermal growth could be seen after 21 days in the control group. The most significant difference between the group treated with the fibroin dressing and the control group was the healing time. The treatment group healed 7 days faster than the control group.

Their histological findings revealed that the collagen deposition in the treatment group was greater with less inflammation. Neutrophil and lymphocyte infiltration of the treatment group was also compared with the control group. They reported only slight infiltration of white blood cells in the treatment group compared with severe infiltration in the control group. They concluded that silk based dressings offer advantages over other dressings and may be clinically useful for wound treatment (Sugihara *et al*. 2000).

Similar results were found in this study. In figure 5.1 red granulation tissue is visible around the edges of the burn wound treated with the novel silk protein based wound dressing (See figure 5.1 1b). In human subjects second degree burns, or partial thickness burn wounds penetrate the dermis of the skin and

therefore do not have hair follicles in tact that can donate epithelial cells. Therefore second degree burn wounds heal by migration of the epithelial cells from the wound edges. In first degree burns or superficial burn wounds, the hair follicles are still intact and epithelial cells can migrate from these hair follicles. First degree burn wounds usually have a red appearance and heal within 5 to 7 days. (Bishop, 2004). No literature is currently available for Wistar rats. Therefore it is expected that the wounds created by the method described in this thesis, partial thickness burn wounds, will heal by means of migration of the epithelial cells from the wound edges, as seen in figure 5.1 1b.

When comparing the treatment group (figure 5.7 and 5.10) with the control group (figure 5.8, 5.11a and 5.11b), the burn wounds of the treatment group showed granulation tissue formation while the burn wounds in the control group showed no granulation tissue formation after 7 days. A representative micrograph of each group is shown.

When comparing the H & E stained histological sections of these two groups the epithelialization is visible in the treatment group (see figure 5.5) while it is not obvious in the control group wound (see figure 5.6). Neovascularization is also evident in the treatment group (see figure 5.7), while it is not visible in the control group (see figure 5.8). These findings are similar to that documented Aramwit and Sangcakul (2007).

The sections stained with Masson's Trichrome revealed that the epithelialization is once again visible in the treatment group (see figures 5.9a and 5.9b) while this is not the case in the control group (see figures 5.11a and 5.11b). Neovascularization is also evident in the treatment group (see figure 5.10); while it is not visible in the control group (see figures 5.11a and 5.11b). White blood cell infiltration is also visible in the control group (see figures 5.11a and 5.11b).

Masson's Trichrome staining is frequently used to evaluate or compare collagen deposition, staining collagen dark blue. Aramwit and Sangcakul (2007) found that sericin improved collagen deposition. Sugihara had similar results when evaluating fibroin's effect on collagen deposition (Sugihara *et al*. 2000). An increase in collagen deposition in the dermal tissue was not visible in this study when comparing the sections stained with Masson's Trichrome of the treatment group (see figure 5.9a) with that of the control group (see figures 5.11a and 5.11b). However, the study done by Aramwit and Sangcakul (2007) ran for 15 days, and that of Sugihara (2000) for 21 days. The study discussed in this thesis only ran for 7 days. Collagen deposition starts between 4 and 5 days after injury and continues at a maximal rate for 2 to 4 weeks. Therefore, the increase in collagen deposition is only visible at a later stage of healing (Monaco and Lawrence, 2003).

5.5. CONCLUSION

In conclusion, it is visible that the novel silk protein based wound dressing improves healing at a microscopic level. At this level it is visible that the novel silk protein based dressing facilitates granulation tissue formation as well as epithelialization. These findings are also supported by the literature.

However, when evaluating collagen deposition by Masson's Trichrome staining, the findings of this study differ from that of the literature. No difference could be seen between the treatment group and the control group regarding collagen deposition. It is known that collagen deposition, during wound healing, starts 4 to 5 days after injury. Therefore, even though no increase in collagen deposition was visible when comparing the treatment group and control group after a 7 day healing period, an increase would be expected at a later stage of wound healing. Masson's Trichrome stain did show white blood cell infiltration in the control group, this is also supported by the literature.

Also, a visual conclusion during the study was made that the novel silk protein based dressing visibly improves granulation tissue formation, additionally supporting the histology.

Chapter 6: Assessing the systemic effect using blood cell counts and scanning electron microscopic analysis of the formation of fibrin networks in the burn wound model treated with the novel wound dressing

6.1. INTRODUCTION:

It is well known that thermal injury has a systemic effect giving rise to complications such as a high incidence of hypercoagulability and prevalence to thrombosis (Kowal-Vern *et al*., 2000). Also pulmonary embolism, adult respiratory distress syndrome, infection and multiple organ failure are common in thermal injury (Gando *et al*., 1992; Geerts, 1994; Penner, 1995; Darling *et al*., 1996; Kowal-Vern *et al*., 2000).

It has been documented that burn injury induces the dermal production of proinflammatory mediators, resulting in ongoing wound inflammation and tissue edema. Additionally, severe burns are known to induce the systemic inflammatory response syndrome, which is associated with a high risk of organ failure (Ipaktchi *et al.,* 2006). It is also known that injury due to burn has an impact on coagulation and haemostasis (Lavrentieva *et al*., 2008) by inducing subclinical disseminated intravascular coagulation.

Predominantly, fibrinolysis is affected. Fang and co-workers (1997) showed that parameters in plasma, 2 hours after thermal injury, shows an activation of fibrinolysis followed by suppression in fibrinolysis after 24 hours until as late as 10 days post-burn. It is thought that this suppression of fibrinolysis may protect the fibrin deposited in the wound, and that this could be important in wound healing. The authors suggest that the suppressive factors of fibrinolysis might be due to

enhanced activities of proteins including plasminogen activated inhibitor (PAI) and alpha 2-antiplasmin after injury.

Furthermore, it has been documented that burn injury causes an acquired deficiency of the plasma protein Antithrombin III (ATIII). ATIII is a natural anticoagulant which causes burn patients to have a high incidence of hypercoagulability and prevalence to thrombosis (Kowal-Vern *et al*., 2000). ATIII is the most important inhibitor of blood coagulation due to its effect on thrombin (Penner, 1995; Kowal-Vern *et al*., 2000). Low plasma levels of ATIII are associated with venous thrombosis known to occur in burn patients. In 2004 Ravindranath and co-workers (2004) also found changes in plasma tissue factor pathway inhibitor (TFPI). They found that TFPI decreased significantly at 24 hours post-burn and thrombin activatable fibrinolytic inhibitor (TAFI) levels increased significantly at 24- and 72-hour post-burn.

Regarding silk, it has been documented that the silk fibroin protein is able to bind to components of the clotting cascade such as fibrin and fibrinogen. This causes silk to be highly thrombic. However, in most cases, this response is moderate and subsides with time (Altman *et al*., 2003).

Additionally, silk, like most proteins, is a potential allergen, causing a type I allergic response in some cases. This indicates that silk may have an effect on the immune system. Silk is composed of two protein components, sericin and fibroin. The novel silk protein based wound dressing contains both these proteins. Literature suggests that the sericin component is the most likely cause of the development of a T-cell mediated allergic response or sensitization. However, fibroin degradation products could also potentially be involved in adverse biological responses (Altman *et al*., 2003). Uff *et al.* showed that soluble factors of silk fibers, the fibroin component, induced pro-inflammatory cytokine production and increased phagocytosis (Uff *et al*., 1995) while Santin *et al.* determined that fibroin membranes elicit low levels of macrophage activation (Santin *et al.,* 1999).

The sericin component is thought to be the main allergenic component in silk. However in a study done by Aramwit and Sangcakul (2007), evaluating the effect of sericin cream on thermal injury in rats, they found no rash or redness on the sericin treated wounds when compared to the control. They also found that the sericin treated wounds healed without any allergic reaction and little to no inflammation while the control wounds still showed signs of inflammation. Therefore, there is some controversy in the literature regarding the allergenic properties of silk proteins.

In the current chapter the Wistar rat model was used to evaluate the systemic effects of the novel silk protein based wound dressing. The first question that arises is whether the silk protein based wound dressing will affect the white blood cell counts in blood smears. The second question that arises is whether the novel silk protein based wound dressing has an impact on fibrin network architecture in circulating plasma, and whether a local burn injury treated with the novel wound dressing will change clot ultrastructure.

6.2. MATERIALS AND METHODS:

6.2.1. Implementing the burn wound model:

Nine female Wistar albino rats weighing between 200 – 250g, maintained at the Uiniversity of Pretoria Biomedical Research Centre were used in this study. The rats were kept in a room with a 12h light and dark cycle and a room temperature constant at 22 °C. The rats were fed a standard rat diet, water *ad libitum* and were housed in individual polycarbonate cages. The rats were fasted for 12h before thermal injury; but they had free access to water. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee.

This study was concerned with the effect of a novel silk protein based wound dressing on wound healing. All nine rats in this part of the study received Buterphanol as part of the analgesic regimen. One group of six rats received treatment with a novel silk protein based wound dressing. The control group, consisting of three rats, was not treated with the novel silk protein based wound dressing. As a negative control each rat of the treatment group served as its own control before thermal injury.

6.2.2. Thermal injury

On the first day (day 0), the rats were anaesthetized with Isoflurane and directly afterwards the analgesic, Tramadol (10 mg/kg), was injected intramuscularly. After injection of the analgesic, 15 minutes was allowed before wound creation in order to allow the analgesic to take effect. The dorsum of each rat was shaved and then exposed to a 1 x 1 cm brass block for 10 seconds (Gottrup *et al.*, 2000; Dorsett-Martin and Wysocki, 2008). This brass block was heated up to 95 °C using a hot water bath. Using gravity only, the brass block was rested on the dorsum of the rats, which resulted in partial thickness skin burns (Mogford and Mustoe, 2001; Dorsett-Martin and Wysocki, 2008). A physiological saline solution was then administrated intraperitoneally (25 mg/kg) in order to prevent dehydration of the animal.

6.2.3. Treatment

Immediately after thermal injury all wounds were dressed with a gauze primary dressing and Opsite® as the secondary dressing. In order to keep the rats from interfering with the healing process the secondary dressing was further covered with a third dressing of bandage and fastened with Elastoplast® around the edges.

On day 3 post-wound creation the rats were divided into two groups. During the dressing changes one group of three rats received the normal dressing changes composed of gauze and Opsite®. This was the control group. Another group of six rats received the normal dressing change composed of gauze and Opsite®.

However their wounds were treated with an additional novel silk protein based primary wound dressing.

6.2.4. Techniques of the blood smears:

A blood sample of each treatment group was taken before burn wound creation to serve as a negative control. A blood sample of each treatment group rat as well as each control group rat (positive control) was taken by cardiac puncture on the day of termination (7 days post-burn). Blood smears were prepared and stained with Rapid Heamatological stain, this was done in triplicate. Therefore, three blood smears were counted for each rat. Lymphocytes, monocytes, neutrophils, eosinophils and basophils were counted and a total of 100 leukocytes were counted for each blood smear. White blood cells were counted using a light microscope at a 400x magnification.

6.2.5. Preparation of the fibrin clots:

On the day of termination (day 7) blood samples were drawn from each rat by cardiac puncture and 11 µl of citrate was added for every 100 µl of blood drawn. The blood from each animal was kept separately and studied individually. The blood samples were then centrifuged at 1250 rpm for two minutes to obtain platelet rich plasma (PRP).

In order to prepare fibrin clots, human thrombin (provided by the South African National Blood Service) was added to the PRP (Pretorius *et al.*, 2007, 2009a,b). The thrombin (20 U/ml) is made up in a biological buffer containing 0.2% human serum albumin. After thrombin is added to the PRP fibrinogen is converted to fibrin. Additionally, intracellular platelet components such as transforming growth factor, platelet derived growth factor and fibroblastic growth factor are released into the coagulum.

Onto a 0.2 µm millipore membrane 10 µl of rat PRP was added onto one side and 10 µl thrombin onto the other side. The two substances were then mixed on the 0.2

µm millipore membrane. This was done to form the coagulum or fibrin clot on the membrane. The millipore membrane was then placed in a Petri dish on filter paper that was dampened with phosphate buffered saline (PBS) in order to create a humid environment. The Petri dish containing the millipore membrane was then placed in an oven at 37°C for 10 minutes. In order to remove any blood proteins that may be trapped in the fibrin network. The millipore membrane with the fibrin clot was then washed by placing it in PBS and magnetically stirred for 120 minutes (Pretorius *et al*., 2007; Pretorius *et al*., 2009b and Pretorius and Oberholzer, 2009).

6.2.6. Preparation of the washed fibrin clots for SEM

After washing the fibrin clots, they were fixed in 2.5% glutaraldehyde in Dulbecco's phosphate buffered saline (DPBS), pH 7.4 for 60 minutes. Each fibrin clot was washed three times in phosphate buffer for 5 minutes and then fixed in 1% Osmium tertraoxide for 60 minutes. The fibrin clots were again washed three times for 5 minutes each, but with distilled water. Serial dehydration was then performed on the fibrin clots in 30%, 50%, 70%, 90% and three changes of 100% aqueous ethanol solutions. The scanning electron microscopy preparation procedure was then completed by drying the samples with hexamethyldisilazane (HMDS) (Araujo *et al.*, 2003), mounting the samples and coating them with ruthenium tetraoxide (RuO_4). The fibrin clots were viewed using a JEOL 6000F FEG scanning electron microscope (Pretorius *et al*., 2007; Pretorius *et al*., 2009b and Pretorius and Oberholzer, 2009)

6.2.7. Statistical analysis

The treatment group and positive control was compared using the student's t-test or unpaired t-test. Since the treatment group also served as its own control (negative control), the paired t-test was used to determine statistical significance. In both instances differences were considered statistically significant when the p-value was smaller than 0.05, resulting in a confidence interval of 95% (www.graphpad.com).

6.3. RESULTS

Table 6.1 shows the average number of white blood cells counted in the blood smears of the treatment group. Table 6.2 shows the average number of white blood cells counted in the blood smears of the negative control group. Table 6.3 shows the average number of white blood cells counted in the blood smears of the positive control group. For each individual rat three blood smears where counted, if possible, and for every blood smear a hundred cells were counted.

Table 6.1: Average number of white blood cells counted in the blood smears of the treatment group (100 cells counted).

	Rat 1	**Rat 2**	**Rat 3**	**Rat 4**	**Rat 5**	**Rat 6**
Lymphocyte:	65.33	65.67	58	25.5	58.33	68.67
Monocyte:	16.67	18.33	18	38	23.33	15.67
Neutrophil:	16	11.33	17	29	13.33	13.33
Eosinophil:	1.67	4.33	6	4	4.33	1.67
Basophil:	0.33	0.33	1	3.5	0.67	0.67

Table 6.2: Average number of white blood cells counted in the blood smears of the negative control group (100 cells counted).

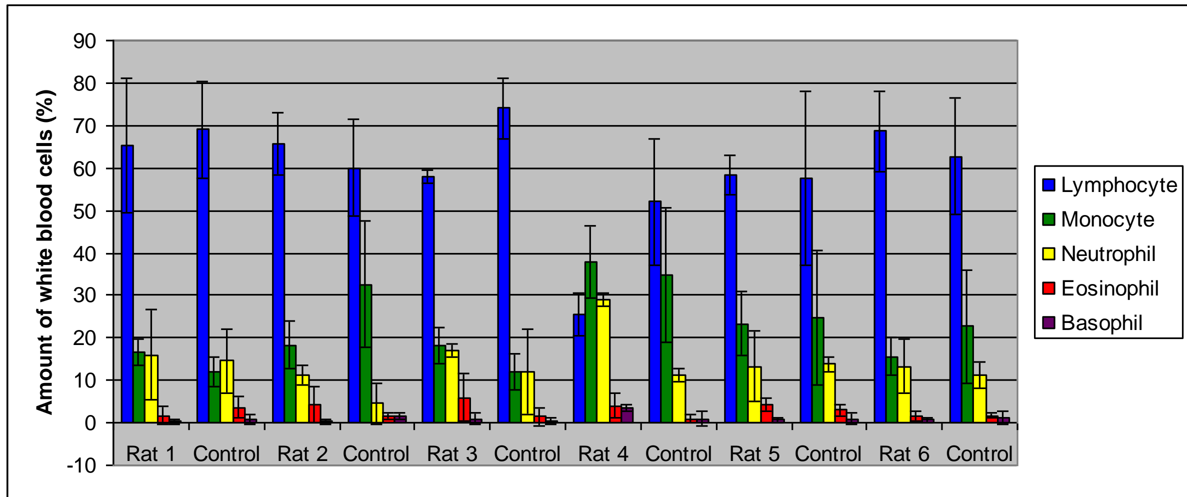
	Rat 1	**Rat 2**	**Rat 3**	**Rat 4**	**Rat 5**	**Rat 6**
Lymphocyte:	69	60	74	52	57.5	62.67
Monocyte:	12	32.5	12	34.67	24.75	22.67
Neutrophil:	14.67	4.5	12	11.33	13.75	11.33
Eosinophil:	3.67	1.5	1.5	1	3	1.67
Basophil:	0.67	1.5	0.5	1	1	1.33

Table 6.3: Average number of white blood cells counted in the blood smears of the positive control group (100 cells counted).

	Rat 1	Rat 2	Rat 3
Lymphocyte:	52	57.5	32
Monocyte:	24.5	17.5	40.5
Neutrophil:	18.5	18.5	21
Eosinophil:	2.5	3.5	4.5
Basophil:	2.5	3	2

Graph 6.1 shows the comparison between the treatment group and the negative control group regarding the white blood cell percentages. Each rat served as its own control. Where possible three blood smears per rat were counted and the average was calculated. T-tests performed, revealed that there was no significant difference between these two groups. Graph 6.2 shows the comparison between the treatment group and the positive control group regarding the white blood cell percentages. Student's t-test performed showed a statistically significant difference in the neutrophil and basophil count with a p-value < 0.05. Three blood smears per rat were counted, where possible, and the averages calculated.

Graph 6.1 The percentage of white blood cells for each rat. A comparison between the treatment group and the negative control group.



Graph 6.2 The percentage of white blood cells for each rat compared with the positive control group.

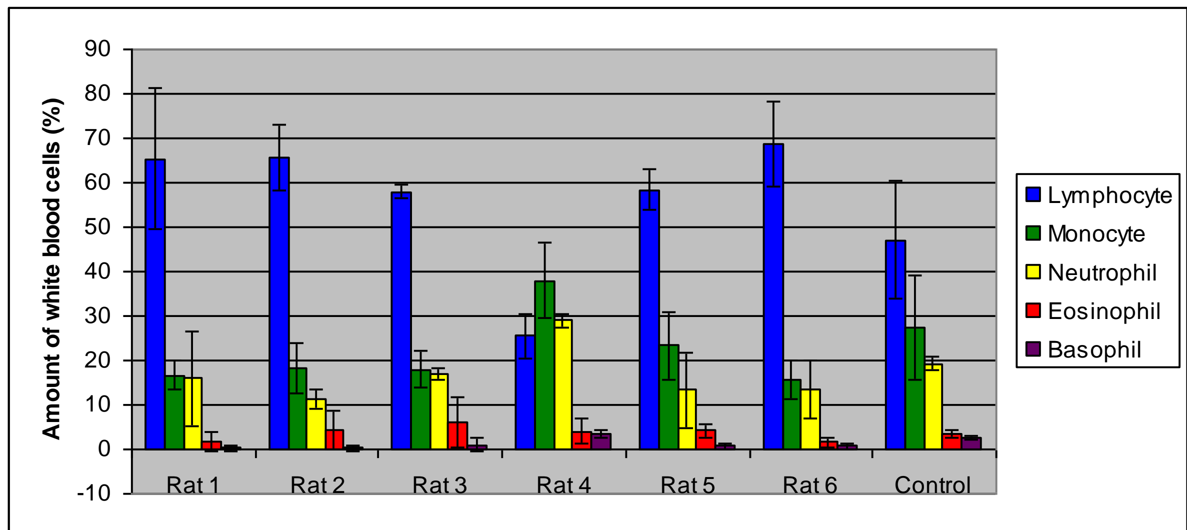


Figure 6.1 shows the control rat fibrin network indicating major, thick fibers and minor thin fibers. Figure 6.2a–c shows the rat fibrin network 7 days after thermal injury indicating major thick fibers and minor thin fibers with a matted appearance.

Figure 6.1: Control rat fibrin network. Thick, white arrow = major, thick fibers; thin, white arrow = minor, thin fibers. Scale = 1 μm.

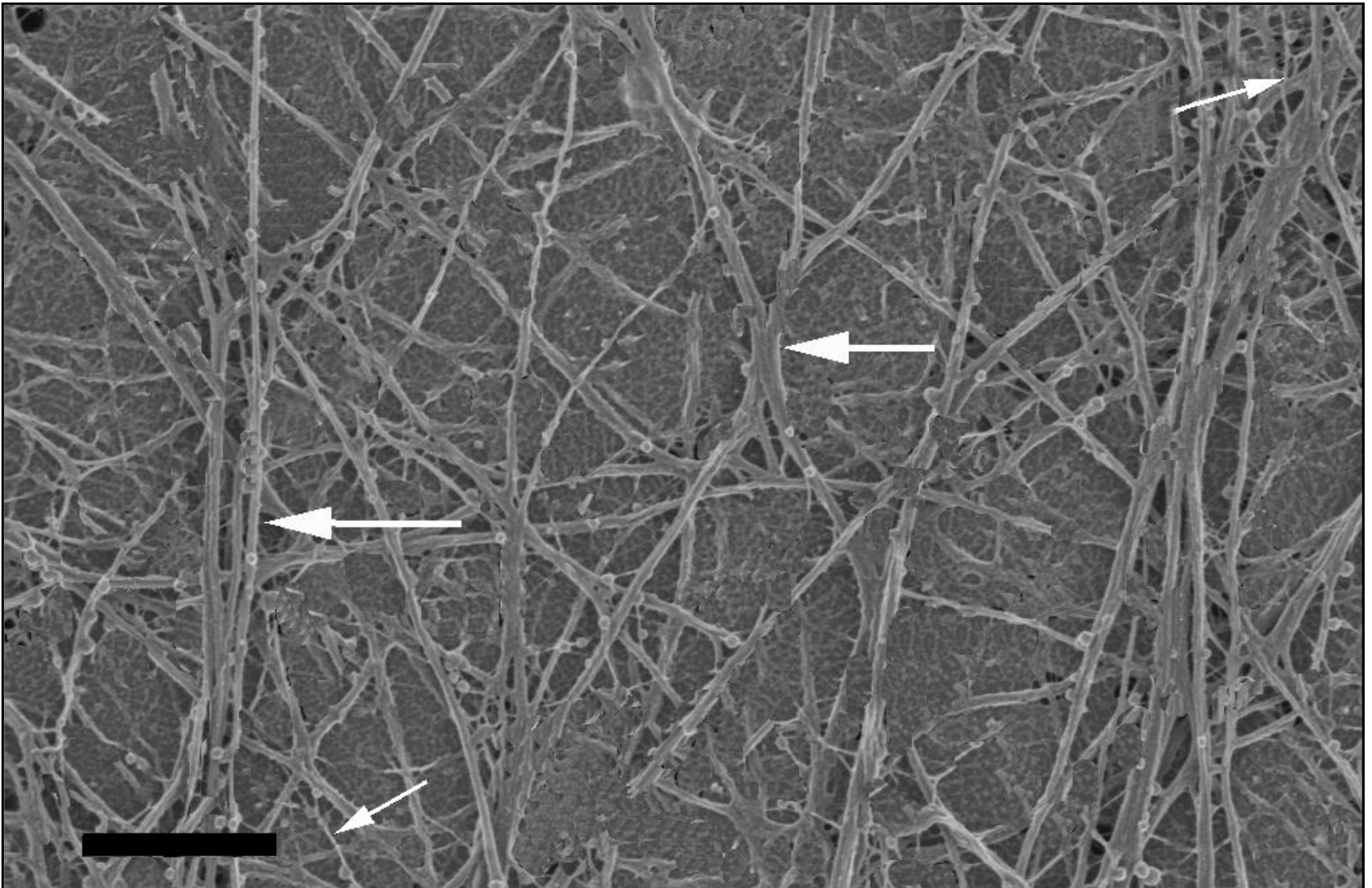


Figure 6.2a: Rat fibrin network on day 7 after thermal injury. Thick, white arrow = major, thick fibers; thin, white arrow = minor, thin fiber layer with netted appearance, found between thick fibers. Black arrow = matted fibrin.
Scale = 1 μm.

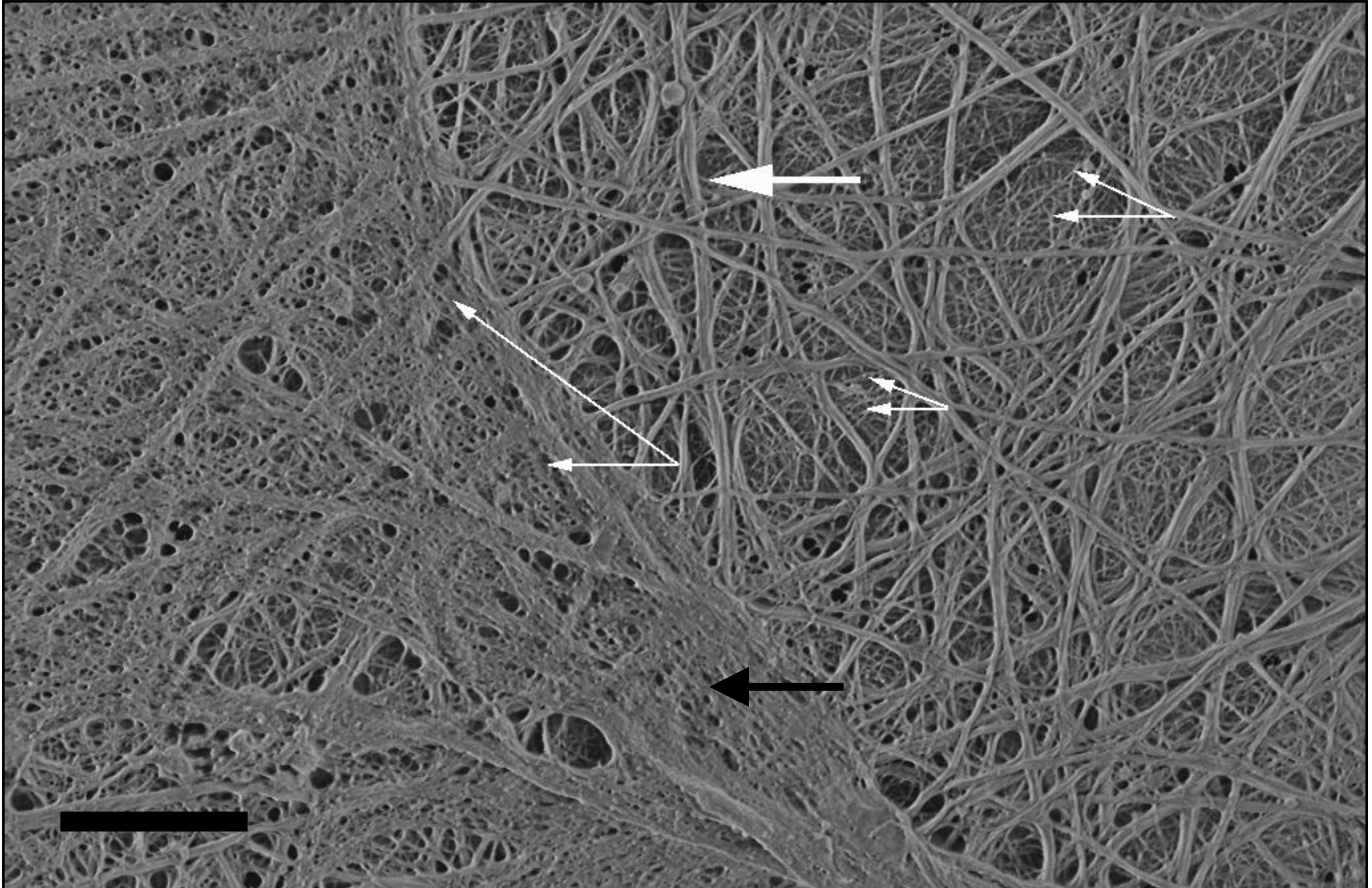


Figure 6.2b: Rat fibrin network on day 7 after thermal injury. White arrow = thickened, matted fibrin. Scale = 1 μm.

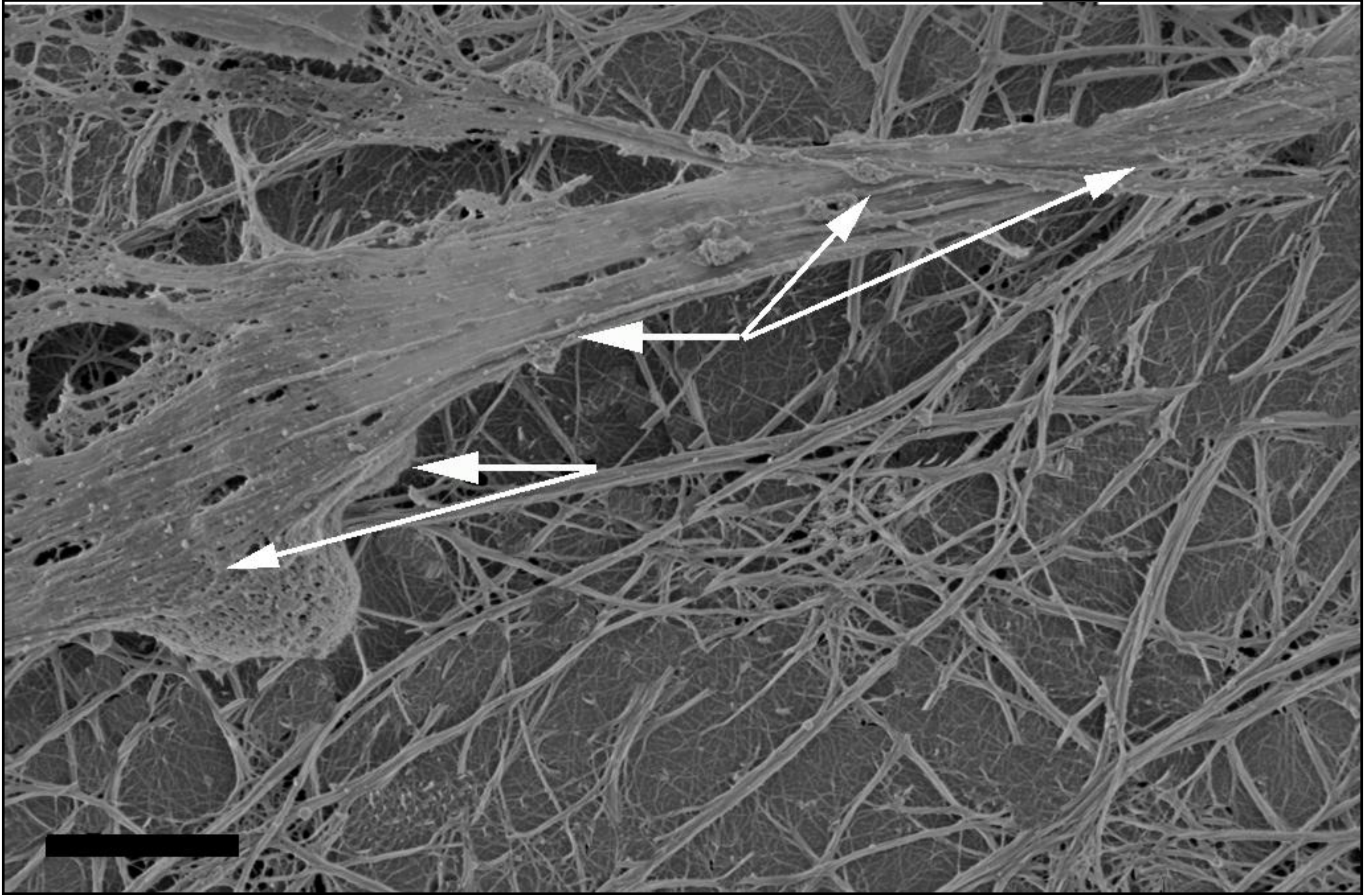
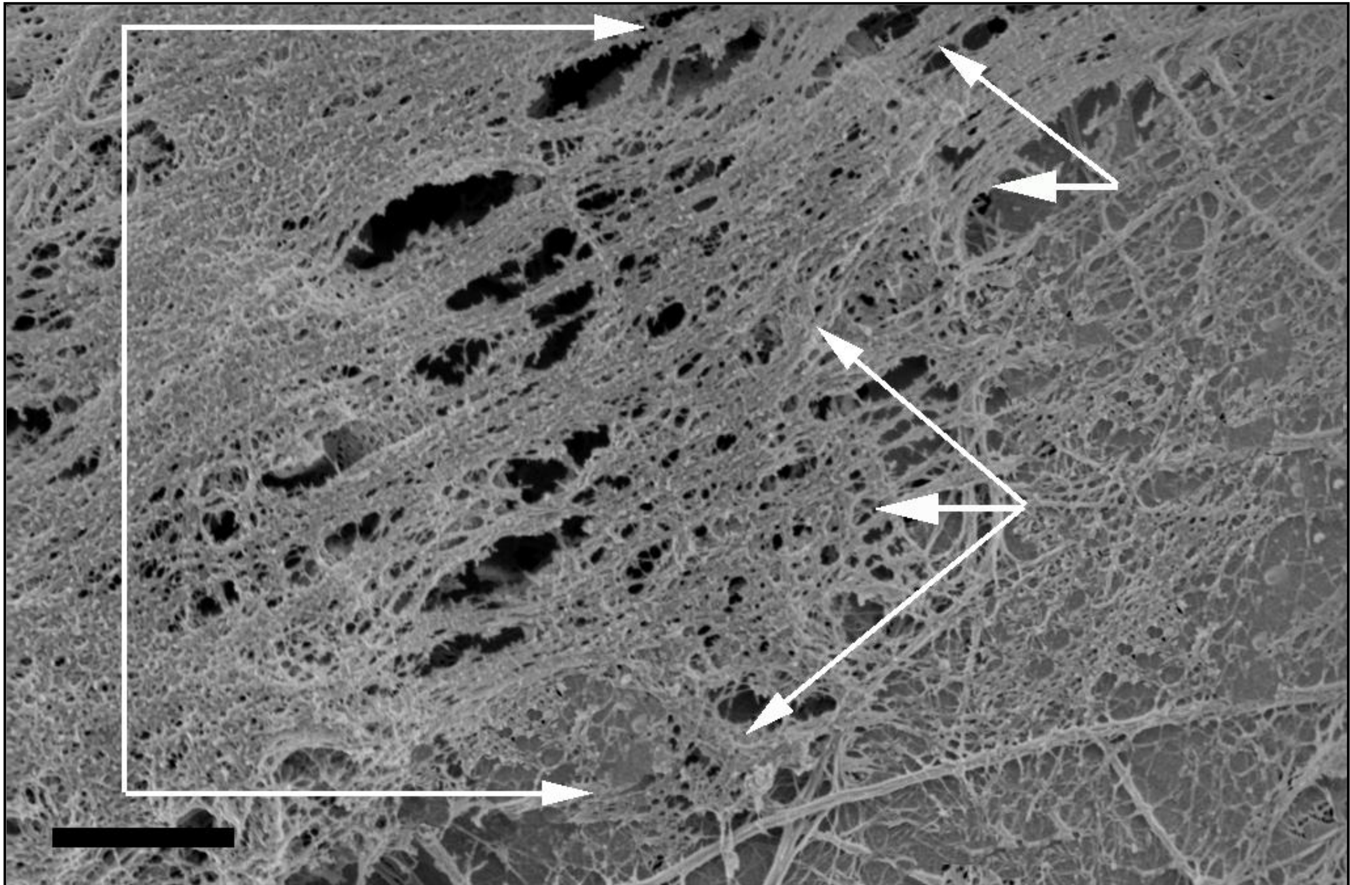


Figure 6.2c: Rat fibrin network on day 7 after thermal injury. White arrow = minor, thin fiber layer with netted appearance. Scale = 1 μm.



6.4. DISCUSSION

According to literature there is a synergistic response between inflammation and the coagulation systems (Sherwood and Toliver-Kinsky, 2004; Park *et al.,* 2008). During the inflammatory response, there is also a changed white blood cell distribution. This changed white blood cell profile in inflammation was also previously shown by Oberholzer and co-workers (2009). Burn injury results in an inflammatory response (Sherwood and Toliver-Kinsky, 2004; Park *et al.,* 2008).

It has been documented that burn injury induces the dermal production of pro-inflammatory mediators. This induction of pro-inflammatory mediators results in ongoing wound inflammation and tissue edema (Ipaktchi *et al.,* 2006). Animal studies have also shown that the inflammatory response in severe burn injury effects leukocyte count and function. Thermal injury causes an inflammatory reaction that can be characterized by an increase in the number of circulating neutrophils as well as neutrophil activation (Maung *et al*., 2008; Suber *et al.,* 2007). In the present study no significant difference could be found between the treatment and the negative control group concerning the white blood cell count in the blood smears, as shown in graph 6.1. However when comparing the negative control group, which received no thermal injury, with the positive control group, which received thermal injury without treatment with the novel silk protein based dressing, a statistically significant difference with a p-value of 0.0137 was found. This verifies that thermal injury through the method used, did cause inflammation.

As mentioned previously, haemostasis is changed during burn injury. Following burn injury, due to intensification of the procoagulant activity, there is a brief hypocoagulation phase, followed by hypercoagulation and this results in depression of fibrinolysis (Kirillov and Alekaeva, 1975). Literature shows that both thrombotic and fibrinolytic pathways are directly triggered proportionally to the extent of the burn injury (Bartlett *et al*., 1981; Wells *et al*., 1984; Opal, 2000). This has been researched thoroughly in the past; however we do not know what the impact of burn injury is on the ultrastructure of the fibrin networks.

Previous research done by Pretorius and co-workers (2007) indicates that inflammation causes fibrin networks to present with a changed morphology. Under normal conditions, control fibrin networks consist of major, thick fibers forming the majority of the clot. Minor, thin fibers are sparingly distributed among the major fibers (Pretorius et al., 2009a,b). It is also known that this arrangement is found both in humans, rabbits and mice, while the thickness of rodent fibrin differs considerably from that of humans (Pretorius et al., 2009b). Inflammation in humans and mice has

previously been shown to look similar, although the fiber thickness varies. Pretorius and Oberholzer (2009) showed that inflammation in humans and mice show major, thick fibers covered by a thin, matted layer of minor, thin fibers.

In the current study, when comparing the fibrin network of the positive control group, receiving thermal injury, and the treatment group receiving treatment with the novel silk protein based wound dressing after thermal injury, no significant difference could be seen. Therefore the observed difference between thermal injury and no thermal injury, as the control, will be discussed in this dissertation.

In the current study, the control rat fibrin networks were found to look similar to that of BALB/c mice; with major, thick fibers and minor, thin fibers distributed in between the major fibers. See figure 6.1. In the burn wound injury group, 7 days after injury, fibrin fibers show a typical inflammation profile. See figure 6.2a - c. Figure 6.2a shows major, thick fibers (thick, white arrow) and more prominent minor, thin fibers (thin, white arrow), also areas where fibrin has a netted appearance (black arrow). Figure 6.2b shows areas of the clot where thickened, matted fibrin is present (white arrow). Areas of the clot also have a netted appearance. See figure 6.2c (white arrow).

There is a visible change in morphology when comparing the burn injury group with the control group. This suggests that thermal injury affects not only the local area where the wound was created, but also more broadly the inflammatory processes and ultimately coagulation and haemostasis. This is also visible in the white blood cell counts (Table 6.1, 6.3 and graph 6.2) as well as the changed fibrin clot structure.

Ravindranath and co-workers (2004) stated that burn injury impairs fibrinolysis, this in turn disturbs the coagulation cascade and thrombotic process toward the procoagulant pathway by impairing fibrinolyses. Also, venous thrombosis, pulmonary embolism and hypercoagulability, are common in thermal injury (Gando *et al*., 1992; Geerts, 1994; Penner, 1995; Darling *et al*., 1996; Kowal-Vern *et al*., 2000). From this study it may be suggested that the thrombotic events associated with burn injury are

due to the thickened, netlike areas formed when thrombin activates the coagulation cascade. Due to the impaired fibrinolysis activities, the resulting fibrin clots cannot be successfully disseminated. It is possible that small fragments of these netted, clumped areas may break loose, and due to an already insufficient fibrinolysis activity, be the cause of thrombotic events during burn injuries.

From the results it is clear that there is a difference between the fibrin network formed after thermal injury and those formed under normal conditions. Even though no differences could be seen between the treatment group and the positive control regarding the fibrin network, some changes in white blood cell counts were noted. See graph 6.2.

According to literature, sericin has certain advantages as a wound dressing. Sericin has been documented to have antibacterial and antioxidant properties (Aramwit and Sangcakul, 2007). Tsubouchi (2005) also concluded that sericin enhanced the attachment of human skin fibroblasts. Sericin also has good compatibility and biodegradation according to Aramwit and Sangcakul. They found that sericin has wound healing effects without causing allergic reactions. They also stated that sericin treated wounds showed no inflammation while the control group still showed some signs of inflammation (Aramwit and Sangcakul, 2007).

In the current study the white blood cell counts in the blood smears of the treated group is compared with that of a positive control group (see graph 6.2). Overall there is a small but statistically significant decrease in neutrophil and basophil count.

Neutrophils are phagocytic cells. They are present in large number at the site of inflammation. They have the ability to move in between the endothelial cells of blood vessels and phagocytose all undesirable particles in the surrounding connective tissue (Coetzee *et al.*, 2003). After tissue damage, such as thermal injury, the innate immune response is the first immune response of which neutrophils form an integral

part. At the site of inflammation, TNF-α initiates an immune response that activates anti-microbial defense mechanisms. It is also a strong activator of neutrophils and mononuclear phagocytes. Acute inflammation after tissue damage is beneficial and forms part of the normal healing process. However, excessive inflammation may cause tissue damage and severe inflammation may even cause systemic inflammatory response syndrome (Sherwood and Toliver-Kinsky, 2004; Park *et al.,* 2008).

Graph 6.2 shows a statistical significant decrease in neutrophil count, with a p-value of 0.0319, when the treatment group is compared to the positive control group. This indicates that the novel silk protein based wound dressing does have some anti-inflammatory effect. This finding is supported by the findings of Aramwit and Sangcakul (2007), stating that serecin cream treated rats were observed to have less inflammatory reactions than the control group. Unfortunately the authors did not produce a table stating the exact blood counts so we could not compare our results with theirs.

Basophils are also phagocytic and play an important role in both allergic and inflammatory reactions (Coetzee et al., 2003). The comparison between the treatment group and the positive control group also show a statistically significant decrease in basophil count with a p-value of 0.0004. Additionally no statistically significant difference could be found when comparing the eosinophil and monocyte counts. Eosinophils play a role in allergic reactions. They are also able to move in between endothelial cells of blood vessels and phagocytose antigen-antibody complexes. Monocytes can form pseudopodia and are therefore mobile. Once they have moved out of the bloodstream and into the connective tissue it is known as a macrophage (Coetzee et al., 2003). Aramwit and co-workers (2008) states in their article that the activation of the innate immune response, specifically macrophages, is a good indication of a biomaterial's biocompatibility.

Aramwit and co-workers (2008) studied the effect of silk sericin by using an *in vitro* macrophage and monocyte assay to assess cytokine activation**.** This was determined by measuring the production of TNF-α and interleukin-β1 (IL-β1). They found that both the monocyte and macrophage cell line produced these inflammatory mediators, but not at a level sufficient to initiate an inflammatory response. Additionally they evaluated the TNF-α and IL-β1 levels *in vitro* as well, making use of a rat model. They found that the sericin treated rats showed much lower levels of both mediators (Aramwit *et al*., 2008).

The results from the current study also indicate that monocyte activation was not induced. There is no statistically significant difference in monocyte count between the control groups and treatment group even though monocyte count is more, in all groups, than what is reported in the literature (Pass and Freeth, 1993). This was double blind checked by three observers. Additionally, neither eosinophil nor basophil count increased, therefore it may be concluded that the novel silk protein based wound dressing does not induce an allergic reaction and may be considered as a biocompatible biomaterial. Also, the novel silk protein based wound dressing may have anti-inflammatory properties.

6.5. CONCLUSION

In conclusion, it was found that after primary exposure to the wound dressing there are no signs of allergy to the novel silk protein based wound dressing. Additionally, the dressing may have some anti-inflammatory property when considering the white blood cell count. However, inflammation was still present to some degree as shown by the observations of the fibrin networks.

Even though no systemic allergic reaction was noted in this study, literature does suggest that patients may be sensitized to silk proteins during the primary exposure and a secondary exposure to silk proteins may trigger a type I allergic reaction.

Therefore, further research must be done to examine whether secondary exposure to the novel silk protein based wound dressing may elicit an allergic response.

After examining the morphology of the fibrin networks of the thermal injured group and comparing it with the control. It may be concluded that thermal injury affects not only the local area where the wound was created, but also more broadly the inflammatory processes and ultimately coagulation and haemostasis. This may be one of the causes of the complications regarding coagulation and haemostasis seen in burn wound victims.

Chapter 7: Concluding discussion

Even though silk has been used as a biomaterial for decades, it has only recently been appreciated for its wound healing properties (Altman *et al*., 2003). It has been reported that silk proteins have photoprotectant and tumour inhibiting properties (Zhaorigetu et al., 2003). Sericin has been documented to have anti-inflammatory properties while both sericin (Aramwit and Sangcakul, 2007) and fibroin have been documented to improve epithelialization and collagen deposition in wound treatment (Sugihara *et al*. 2000). Copper ions have also been documented to improve wound healing (Nasulewicz *et al*., 2004; Gupte and Mumper, 2009; Borkow *et al*., 2008; Sen *et al*. 2002).

Although studies have been done to evaluate the effect of silk proteins on wound healing, no research has been done on the effect of silk proteins in combination with copper ions. Additionally, there are few studies on the effect of thermal injury on clot ultrastructure and none on the effect of silk proteins on clot ultrastructure. To investigate the effect of these two actives, silk proteins and copper ions, on burn wound healing it was necessary to establish a Wistar rat burn wound model. This was done by comparing the effectiveness of Morphine and Butorphanol for a 7 day study. The general clinical signs and behavioural signs of pain were documented. From the results it was evident that both Morphine and Butorphanol, injected subcutaneously, was effective as an analgesic for a 7 day burn wound study using the Wistar rat as the animal model for thermal injury. However due to the sedative effect of Morphine (Masica *et al*., 2007), Butorphanol proved to be the better choice.

Before the established Wistar rat burn wound model could be used to evaluate the effect of the novel silk protein based wound dressing on the healing of a partial thickness burn wound, it was necessary to determine whether this dressing has cytotoxic effects. The results showed that the dressing was not cytotoxic even at a concentration of 25 mg/µl since no IC_{50} value could be found. It was therefore concluded that the novel silk protein based wound dressing was not cytotoxic.

After determining that the novel silk protein based wound dressing was indeed not cytotoxic, the established Wistar rat burn wound model was used to investigate the dressing's effect on wound healing, both at the site of application and systemically.

Aramwit and Sangcakul evaluated the effect of sericin after topical application. Their results indicated that sericin has anti-inflammatory effects and did not cause an allergic reaction. They also reported that sericin improved epithelialization and collagen deposition. However they evaluated the effect of sericin on an excisional wound and not a burn wound (Aramwit and Sangcakul, 2007). Sugihara *et al.* evaluated the effect of fibroin on wound healing in 2000. They found similar results adding that fibroin shortened the healing time. However, they too utilized dermatotomies and not burn wounds (Sugihara *et al*. 2000). In the current study similar results were recorded using a Wistar rat burn wound model.

Observations of the burn wounds themselves revealed visible red granulation tissue formation in the treatment group after 7 days. This was not the case in the control. After microscopic investigation by means of H & E staining and Masson's Trichrome staining morphological differences between the treatment group and the control group could also be seen. Epithelialization and neovascularization was visibly improved in the treatment group when compared to the control. Only representative micrographs are shown in this study.

From these results it was concluded that the novel silk protein based dressing improves granulation tissue formation and epithelialization after application to a partial thickness burn wound. However no visible difference could be found between the treatment group and the control group with regards to collagen deposition after 7 days. The Wistar rat burn wound model was also used to evaluate the systemic effects after applying it to a partial thickness burn wound.

Thermal injury has a systemic effect. This can be concluded from the complications seen in such injuries. Complications such as hypercoagulability and a prevalence to thrombosis (Kowal-Vern *et al.,* 2000). Also, pulmonary embolism, adult respiratory distress syndrome, infection and multiple organ failure are common in thermal injury (Gando *et al*., 1992; Geerts, 1994; Penner, 1995; Darling *et al*., 1996; Kowal-Vern *et al*., 2000). Burn injury is also characterized by ongoing wound inflammation and tissue edema caused by the dermal production of proinflammatory mediators (Ipaktchi *et al.,* 2006).

It has been documented that fibrinolysis is affected by thermal injury, possibly due to the up regulation in the expression of proteins such as PAI, alpha 2-antiplasmin and (Fang *et al*., 1997) TAFI (Ravindranath *et al.,* 2004) and down regulation of proteins such as ATIII (Kowal-Vern *et al*., 2000) and TFPI (Ravindranath *et al.,* 2004). Silk has also been documented to be thrombic due to its ability to bind to components of the clotting cascade such as fibrin and fibrinogen. Additionally, it has been reported that silk can cause a type I allergic response (Altman *et al*., 2003). Therefore silk proteins could have a systemic effect.

All of this considered the question arises whether the novel silk protein based wound dressing would have an effect on the white blood cell count as an indication of inflammation and allergic response (Oberholzer *et al*., 2009; Sherwood and Toliver-Kinsky, 2004; Park *et al.,* 2008). After statistically investigating the white blood cell counts, it indicated that the novel silk protein based wound dressing has anti-inflammatory properties. Additionally this analysis indicated that the dressing did not cause an allergic response after primary exposure. The systemic effect of the novel silk protein based wound dressing on fibrin networks was also evaluated.

In the current study, when comparing the fibrin network of the control group, receiving thermal injury, and the treatment group receiving treatment with the novel silk protein based wound dressing after thermal injury, no significant difference could be seen. It was therefore concluded that the dressing does not affect clot

ultrustructure. However, since almost no literature could be found on the effect of thermal injury on fibrin networks and clot ultrastructure, it was decided to discuss the observed difference between thermal injury and no thermal injury.

In the burn wound injury group, 7 days after injury, fibrin fibers showed a typical inflammation profile when compared to the control. Major, thick fibers and more prominent minor, thin fibers, also areas where fibrin has a netted appearance could be seen. Some areas of the clot had thickened, matted fibrin. Therefore, there is a visible change in morphology when comparing the burn injury group with the control group. This suggests that thermal injury systemically affects the inflammatory processes and ultimately coagulation and haemostasis.

After evaluating the white blood cell counts and fibrin network ultrastructure, it was concluded that primary exposure to the novel silk protein based wound dressing does not induce an allergic response and that the dressing exhibits anti-inflammatory properties. It can also be concluded that the dressing does not affect the fibrin network formation in circulating plasma or change clot ultrastructure.

Considering all the results obtained in this study it is concluded that a combination of silk proteins, sericin and fibroin, and copper ions, have a positive effect on wound healing. The actives in the form of a novel silk protein based wound dressing improve granulation tissue formation by improving epithelialization and neovascularization. It was not possible to establish whether the novel silk protein based wound dressing improved collagen deposition within 7 days after thermal injury. Therefore, further research is required to investigate the effect of silk proteins and copper ions in combination, in the form of a silk protein based wound dressing ,on collagen deposition. It is also concluded that this dressing, containing silk proteins and copper ions have an anti-inflammatory effect. However, the actives of the novel silk protein based wound dressing do not systemically affect fibrin network formation or clot ultrustructure and do not cause an allergic reaction after primary exposure.

Chapter 8: References

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