The Anti-Inflammatory Potential of Quercetin and
L-2-Oxothiazolidine-4-carboxylate (OTC)
in Developing Scar Tissue

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Abstract

Loss of physiological function, uncomfortable symptoms and various disease processes are thought to be directly related to the formation of scar tissue following tissue damage. Between ten and thirty percent of patients requiring spinal surgery suffer from failed back surgery syndrome. The pain and instability resulting from failed back syndrome often requires medical treatment and may even require additional surgeries to alleviate its associated symptoms. Following surgery, scar tissue forms that often becomes adherent to the dura and entangled in the ganglia and nerve fibers of the spinal nerves. This scar tissue is considered to play a major role in the development of failed back syndrome.

Following tissue injury, excessive oxidative stress and inflammation are considered to be the primary stimulators behind increased fibroblast proliferation and activation, resulting in abundant extracellular matrix deposition. The excessive laying down of extracellular matrix ultimately leads to abundant scar tissue formation. I hypothesized that reducing oxidative stress and inflammation will mitigate scar tissue formation and produce a better outcome after spinal surgeries. Quercetin is a dietary flavonoid with anti-oxidant and anti-inflammatory properties that has been shown to improve the outcome following injury to the spinal cord and reduce the proliferation of fibroblasts. L-2-Oxothiazolidine-4-carboxylate (OTC) also minimizes inflammation and protects against oxidative stress by promoting the synthesis of the potent antioxidant and anti-inflammatory agent glutathione. OTC reduces airway inflammation in asthma models and is potentially capable of modulating extracellular matrix production.

Treatment with these two agents was hypothesized to decrease oxidative stress and inflammation, thereby causing an amelioration of scar tissue formation following spinal surgery and improve the outcome. Morphological changes observed initially indicated that improvements in wound healing were occurring in the experimentally treated tissues. In addition, the scar tissue area and the lateral widths of the peridural scar forming between the muscular tissue areas suggested a reduction in the scar size. Although inflammatory cell numbers increased slightly in the experimental treatment
groups, particularly during the initial three day post laminectomy time point, this increase was not statistically significant.

While quercetin and OTC did not appear to inhibit the influx of inflammatory cells following laminectomy, they did appear to induce a more beneficial wound healing environment. It is possible that these agents are affecting parameters of wound healing not considered by these studies. For instance the myriad of processes mediated by growth factors and cytokines involved in wound healing process may play a much greater role than the inflammatory cells themselves. In conclusion, reducing oxidative stress and inflammation by these agents to ameliorate scar tissue formation following spinal laminectomy was supported by the observed morphology, but not supported by the quantification of inflammatory cells. Additional studies investigating the efficacy of quercetin and OTC on the wound healing process are needed to further understand the role they play in repair and scar tissue formation.
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<tr>
<td>ECM</td>
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<td>FBS</td>
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1.0 Introduction

1.1 Health Complications Incurred by Scar Tissue Following Surgery or Injury

Scar tissue is the root of many health problems, its formation following injury, surgery, or disease can cause interruptions in normal physiological function. Cirrhosis (Wallace, Burt et al. 2008), idiopathic pulmonary fibrosis (Meltzer and Noble 2008), and sarcoidosis (Wiegand and Brutsche 2006) are just a few of the many disorders caused by or exacerbated by scar tissue formation associated with disease. Injury, burns, or infection resulting in scarring can cause patients to suffer from pain, itching, discomfort, and disability. Additional serious disorders resulting from excessive scarring are contracture and hypertrophic or keloid scarring (O'Leary, Wood et al. 2002). Scar formation plays a major adverse role in spinal surgery resulting in failed back syndrome (FBS).

Failed back syndrome is used to describe the condition of patients having poor outcomes following lumbar spinal surgery for degenerative disc disease (Onesti 2004), and occurs in 10%-30% of patients (Fritsch, Heisel et al. 1996). Sciatic pain, neurological deficiency or lumbar instability leads to re-intervention in 5% - 18% of patients, increasing the rates of epidural fibrosis and lumbar instability as more surgical interventions are required (Fritsch, Heisel et al. 1996). The risks of surgical revision include wound infection and damage to the dura while freeing it from surrounding scar tissue causing spinal fluid leakage. These outcomes must be considered when determining patient treatment and therefore physical therapy and pain management are often considered the preferred treatments. If physical therapy does not produce results within a year, pain management becomes the primary focus with pharmacological interventions or the implantation of spinal cord stimulators or morphine pumps (Onesti 2004). Implantation of these pumps, however, can lead to scar tissue formation that may decrease the efficacy of the pain killer being administered (Protopapas, Bundock et al. 2007). In addition, studies of outcomes of spinal cord injury have reported that spinal tissue scarring may cause a tethering effect on the spinal cord possibly resulting in
significant changes in the spinal cord parenchyma leading to impairing cystic cavity formation (Morikawa, Takami et al. 2006).

The detrimental effects of scar tissue have led investigators to seek ways to reduce its formation. Studies have been conducted investigating both biologic and non-biologic methods to reduce scar formation following spinal laminectomy, including the use of absorbable gelatine sponge, polylactic acid membrane, fat grafts, ligamentum nuchae and ligamentum flavum (Lee, Yang et al. 1990). These studies of different interposing membranes following surgery indicated non-biological membranes were ineffective while biological membranes produced a range of disadvantages making these post-surgical treatments unsatisfactory (Lee, Yang et al. 1990). In addition to these physical barriers, chemical approaches, such as nonsteroidal anti-inflammatory drugs (NSAIDs), were studied as they have the advantage of not introducing foreign materials into the body that may increase the inflammatory response (Sandoval and Hernandez-Vaquero 2008). It has been shown that treatment with an NSAID can reduce scar tissue formation following laminectomy, perhaps due to their ability to inhibit prostaglandin synthesis thereby reducing the permeability of blood vessels and decreasing inflammatory cell migration into the wound (Sandoval and Hernandez-Vaquero 2008). While NSAIDs have been found to promote healthier wound healing, they also produce a number of negative side effects, largely gastrointestinal and renal effects (Reish and Eriksson 2008). Additional studies are required to improve the methods of preventing scar tissue formation following spinal surgery.

1.2 Healing Events Following Injury Possibly Leading to Scar Tissue Formation

Scar tissue formation is related to the immune response, primarily due to the cellular components of the inflammatory response being produced in excess and accumulating in the healing tissue (O’Leary, Wood et al. 2002). When the tissue is injured, a series of events occur including reduced blood flow, increase in vascular permeability, and increased generation of free radicals and calcium overload, resulting in oxidative stress and inflammation. Oxidative stress is the condition whereby the production of free radicals exceeds the organism’s normal scavenging capacity.
Increase in reactive oxygen species promotes the activation of nuclear transcription factors such as Nuclear Factor Kappa B (NF-κB) and cJun leading to the activation of pro-inflammatory genes and eventual transcription of pro-inflammatory molecules (Christman, Blackwell et al. 2000). Consequently, there is an increased production of cell adhesion molecules (e.g. iCAM) and various chemokines, attracting macrophages and other leukocytes to the wounded area. These events all contribute to the establishment of inflammation (Monaco and Lawrence 2003).

The process of wound healing in the skin, which is the most thoroughly studied, is characterized by at least four main stages. The first stage involves vasoconstriction and platelet aggregation in the wound area. The activated platelets and monocytes entering a healing wound shortly after injury will release vascular endothelial growth factor (VEGF) incurring proliferation and increased vascular permeability thereby encouraging blood vessel formation in damaged tissues (Bates and Jones 2003). In addition, in vivo studies indicate that fibronectin concentrations increase around growing fibroblasts and within the walls of newly forming vessels at the time when there is extensive migration and proliferation of cells (Clark 1988). An intense inflammatory process follows this and persists for the first one to three days after injury at which time there is an increase in the concentration of inflammatory cells in the injured area (O'Leary, Wood et al. 2002). An important molecule whose expression is upregulated during this process is Transforming Growth Factor β (TGF-β), which causes fibroblast proliferation and matrix deposition in the wound. This marks the granulation phase of tissue repair that will occur for two to six days following the injury (O'Leary, Wood et al. 2002). It is during this phase that granulation tissue, comprised of connective tissue cells and ingrowing young vessels, is formed within the wound. This tissue matrix is comprised of type III collagen, fibronectin, glucosaminoglycans and other more minor components (O'Leary, Wood et al. 2002). As the matrix is laid down, it becomes richly concentrated with type I collagen in lieu of type III collagen. However, a remaining abundance of fibrogenic cytokines such as TGF-β will prolong the presence of myofibroblasts in the wound and drive the expression of the extracellular matrix molecules (O'Leary, Wood et al. 2002). In conditions of increased inflammation, this fibroblast-mediated matrix deposition occurs in excess resulting in excessive scar tissue formation. Once an abundant matrix has
been laid down, the fibroblasts will conclude their synthesis of type I collagen (Monaco and Lawrence 2003). The final phase in wound healing is the remodeling phase in which scar tissue is formed by the dense masses of granulation tissue connecting the healed wound together (Singer and Clark 1999; O'Leary, Wood et al. 2002).

1.3 Significance of Inflammatory Cell Types in Healing and Scar Tissue Formation

1.3.1 Macrophages

In the few hours after injury platelets release factors attracting leukocytes into the wound site. Neutrophils are the first to arrive, reaching their peak concentrations about 24 hours after injury (Regan 1994; Martin, D'Souza et al. 2003). The primary role of the neutrophil is to function as a phagocyte, clearing the wound of dead and infected cells and other debris. However neutrophils release free radicals resulting in oxidative stress leading to tissue damage (Martin and Leibovich 2005). Following their function of phagocytosis within the wound environment, neutrophils undergo apoptosis and become debris in the wound themselves, needing to be cleared for proper wound healing to occur.

Monocytes enter the site of injury and become activated by the cytokines within the healing wound, converting them to their active form, the macrophage. It is at this point that the macrophage begins replacing the neutrophil as the main phagocytic cell debriding the wound of dead cells and tissues, including the apoptotic neutrophils (Shapiro 2003). Macrophages typically reach their peak concentrations in the wound about 3 days following injury (Regan 1994). Although the macrophage is largely active early in the healing wound, they are thought to play a critical role in modulating the repair process by release of many its factors. They also tend to remain in the wound long after the repair process is complete (Haase, Kirschning et al. 2003; Martin and Leibovich 2005). In studies using PU.1-null mice, mice devoid of neutrophils and macrophages and therefore unable to mount an effective inflammatory response, it has been observed that a slightly enhanced rate of re-epithelialization occurs and no hindrance to the wound healing process ensues (Hopkinson-Woolley, Hughes et al.)
1994; Martin, D'Souza et al. 2003). Thus, healing in the PU.1-null mouse is similar to embryonic healing as it occurs in the absence of fibrosis with a nearly scar free result (Hopkinson-Woolley, Hughes et al. 1994).

Various cytokines, growth factors and mediators of inflammation released by macrophages play key roles in the regulation of fibroblasts and microvasculature, both important in the process of fibrosis and scarring (Martin and Leibovich 2005). In addition human keloid scars exhibit higher numbers of macrophages than normal skin (Boyce, Ciampolini et al. 2001). It is possible that the pro-inflammatory factors released from the macrophage is not entirely necessary for the wound healing process itself, but plays a bigger role in the fibrosis that takes place during the healing process.

1.3.2 T Lymphocytes

T lymphocyte participation in wound healing has been thought to be minimal for many years with studies focused primarily on other leukocytes such as macrophages. It is for this reason that little is known about the exact function of T lymphocytes on wound healing and scar tissue formation. T lymphocytes have been observed to enter a wound later than the phagocytic cells, with their peak concentrations appearing at approximately 7 days following injury (Fishel, Barbul et al. 1987; Regan 1994). High percentages of these cells have been found in adult wounds, as well as fetal wounds treated with TGF-β to induce the inflammatory response, indicating that lymphocytes likely play some role in the wound healing process (Adolph, DiSanto et al. 1993). There are two major subsets of T lymphocytes. The first are the T helper/effector lymphocytes or CD4 T lymphocytes and they function to activate or direct other immune cells during infection and the repair process. The second major subset are the T suppressor/cytotoxic lymphocytes or CD8 T lymphocytes. These suppress activation of the immune system to prevent auto-immune disease and also regulate the immune mediated death of infected or injured cells. Studies have indicated the ratio of CD4 T lymphocytes to CD8 T lymphocytes to be higher at the site of wound repair than in healthy tissues (Fishel, Barbul et al. 1987; Breslin, Wasserkrug et al. 1988). Normal human wounds have an initial high CD4:CD8 ratio that decreases as the healing
process progresses (Boyce, Jones et al. 2000). It was also observed that keloid scar tissue has higher CD4:CD8 T lymphocyte ratios than healthy skin tissue (Boyce, Ciampolini et al. 2001).

The depletion of the T helper (CD4) subset of T lymphocytes was found to have no marked effect on wound healing, whereas depletion of the T suppressor (CD8) subset caused increases in wound breaking strength and collagen synthesis (Barbul, Breslin et al. 1989). Thus indicating that CD8 T lymphocytes may play a role in down regulating the fibrotic wound healing process. In contrast to these earlier studies, Wójciak et al. discovered that the presence of CD4 T lymphocytes or their cytokines within the wound upregulated the healing process (Wojciak and Crossan 1994). Independent depletion of either of these subsets of T lymphocytes appears to have potentially negative consequences on the wound healing. Imbalances in these lymphocyte subsets appear to be what affects the outcome of wound healing.

1.3.3 Mast Cells

The role of mast cells in the wound healing process has yet to be clearly elucidated, however their presence is associated with a broad range of skin conditions including hypertrophic scarring (Sasaki, Mueller et al. 2003). Secretion of their granular contents has the potential to regulate certain aspects of wound healing as mast cells synthesize important mediators such as cytokines, growth factors and proteases, including tryptase and chymase (Metcalfe, Baram et al. 1997). Eishi et. al discovered that a reduction in itching, pain, and fibrosis following treatment of keloid scars with silicone gel sheets coincided with reduced mast cell numbers (Eishi, Bae et al. 2003). In healing myocardial infarcts, mast cells are found to accumulate within the infarcted area and were hypothesized to contribute to fibroblast growth and angiogenesis through the release of tryptase (Somasundaram, Ren et al. 2005). Mast cells have also been shown to accumulate near wound edges with corresponding increases in tryptase and chymase found in the regenerated skin following burn and cutaneous wound studies (Nishikori, Kakizoe et al. 1998; Iba, Shibata et al. 2004). The mast cell proteases, chymase and tryptase, have been shown to promote fibroblast proliferation and connective tissue
organization in addition to their pro-inflammatory and angiogenic abilities (Trautmann, Toksoy et al. 2000; Somasundaram, Ren et al. 2005). Therefore decreases in mast cells may correlate with a more beneficial repair process in damaged tissues.

1.4 Significance of VEGF within the healing tissues

Vascular endothelial growth factor (VEGF) is considered to be the most significant promoter of angiogenesis following injury. Angiogenesis is important in wound healing for delivery of nutrients and oxygen for proliferating cells. Reactive oxygen species (ROS) released in the respiratory burst from infiltrating neutrophils and macrophages promote VEGF production upon injury (Sen, Khanna et al. 2002). Additional release of angiogenic factors including VEGF by infiltrating inflammatory cells, such as the macrophage, further ensure its presence and effects early in the wound healing process (Bates and Jones 2003). In the later stages of wound repair, the fibroblast becomes the predominant cell type within the wound, producing VEGF under hypoxic conditions at such predictable concentrations that the age of wounds can be determined post mortem based on its concentrations (Takamiya, Saigusa et al. 2002).

VEGF within the healing wound functions to increase nutrients provided to the tissues and to facilitate the speedy removal of waste products, doing so through vasodilation, angiogenesis, and increasing vascular permeability (Bates and Jones 2003). However, wounds in the oral mucosa, which heal with less angiogenesis than cutaneous wounds, heal faster with lesser scarring than similar wounds in skin (Szpaderska, Zuckerman et al. 2003). It was found that although a certain level of angiogenesis may be required for healthy wound healing, excessive angiogenesis may actually support scar tissue formation (Szpaderska, Walsh et al. 2005). In addition, Roman et al discovered that treatment with anti-angiogenic agents resulted in decreased angiogenesis in skin wounds, without affecting the quality of healing (Roman, Choy et al. 2002). New vasculature and its associated factors within the healing wound is a vital part of the healing process, but may be detrimental to the healing process if in excess.
1.5 Significance of Reducing the Inflammatory Response within Healing Tissues

Fetal tissues have the unique ability to heal more rapidly and without scar tissue formation following injury. This result has been found to be independent of the fetal environment and considered to be mediated by alternative cellular aspects than that occurring in adult wound healing (Longaker, Whitby et al. 1994). The lack of scarring in fetal tissues is associated with a lack of neutrophil infiltration (Krummel, Michna et al. 1988), reduced macrophage and monocyte infiltration (Adzick, Harrison et al. 1985), as well as reduced angiogenesis at the wound site (Whitby and Ferguson 1991). Yet, the fetus is capable of instigating a full blown inflammatory response following insertion of TGF-β soaked sponges in cutaneous injuries in fetal rabbits (Adolph, DiSanto et al. 1993).

Scar tissue formation is therefore considered by many authors to be the result of an excessive inflammatory response that if attenuated would reduce the undesirable symptoms and loss of physiological function experienced by patients following injury or surgery. Various methods of improving the wound healing environment to reduce inflammation have been investigated with favorable results. Zhang et al showed that hyperbaric oxygen therapy (HBO), a method of increasing oxygen levels within a wound, along with the free radical scavenger N-acetylcysteine attenuated apoptosis and inflammation in ischemic wounds resulting in enhanced wound healing (Zhang, Chang et al. 2008). Curcumin, a major compound in the spice tumeric that exhibits anti-inflammatory and anti-oxidant capabilities, was applied topically to wounds and found to increase the levels of several oxidant scavengers while improving the rate of healing and the tensile strength of the resultant wound (Panchatcharam, Miriyala et al. 2006). Burns heal faster in rats when treated with chamomile extract, previously shown to exhibit anti-inflammatory, anti-oxidant, and anti-microbial properties (Jarrahi 2008).

If the anti-inflammatory and anti-oxidant properties of these treatments have a positive effect on wound healing, a broad range of treatments could have the potential to improve the outcome following injury. Dietary flavonoids, for example, have been suggested as potential mediators of wound healing and inflammatory disease processes as a result of their phase 2 enzyme inducing and anti-inflammatory properties (Juurlink...
and Paterson 1998; Juurlink 2001; Kempuraj, Madhappan et al. 2005). Certain inhibitors of inflammation, such as non-steroidal anti-inflammatory drugs (NSAIDs) like cyclooxygenase-2 inhibitors, are controversial because they have both beneficial and negative effects on wound healing (Reish and Eriksson 2008). A common theme in the literature of improved wound healing seems to be the amelioration of pro-inflammatory events, the inflammatory response itself, and its associated factors to improve the outcome following injury or surgery, not its complete elimination.

1.6 Quercetin

The bioflavonoid quercetin is a strong antioxidant and anti-inflammatory agent. It is also well known as a potent iron chelating agent (Leopoldini, Russo et al. 2006). It has been shown to have a protective effect against ultraviolet induced oxidative stress (Inal and Kahraman, 2000). And it has also been found that the administration of this flavonoid following spinal cord injury in rats can decrease inflammation thereby improving their recovery (Schultke, Kendall et al. 2003). Therefore quercetin may also help regulate tissue repair and scarring.

Quercetin has been shown to inhibit the expression of pro-inflammatory genes by the inhibition of certain MAP kinases that activate the NF-κB and jun kinase (JNK) pathways (Uchida et al., 1999), likely inhibiting these pathways by acting on protein kinases located upstream (Davies, Reddy et al. 2000). Other mechanisms involved in reducing inflammation and oxidative stress may include the competitive inhibition of myeloperoxidase and xanthine oxidase (Pietta, 2000). Quercetin also reduces inflammation via its chelation of iron and oxidant scavenging abilities (Middleton, Kandaswami et al. 2000; Pietta 2000; Zou, Lu et al. 2004).

In vitro studies indicate that fibroblasts from both hypertrophic and keloid scars cease to proliferate when treated with quercetin (Phan, Sun et al. 2003), possibly due to blocking the TGF-β/Smad-signaling pathways (Phan, Lim et al. 2004). The efficacy of quercetin in reducing inflammation and the proliferation of keloid and hypertrophic scar fibroblasts warrants further investigation into its use as a treatment for scar tissue formation following spinal laminectomy.
1.7 L-2-Oxothiazolidine-4-carboxylate (OTC)

L-2-Oxothiazolidine-4-carboxylate (OTC) minimizes inflammation and protects against oxidative stress by promoting the synthesis of the potent antioxidant and anti-inflammatory agent glutathione (Juurlink, Paterson. 1998). OTC is a procysteine compound that is acted upon by 5-oxoprolinase after uptake to give rise to cysteine. Cysteine is the rate limiting amino acid of the three required for glutathione synthesis and thus increase in its abundance will promote the synthesis of glutathione. Glutathione protects cells when they are under the influence of reactive oxygen species (ROS) by acting as an electron donor for a number of enzymes that react with ROS, such as hydrogen peroxide and lipid peroxides (Juurlink 2001).

Procysteine compounds have been found to normalize matrix production in type II alveolar cells from the lungs of ethanol-fed rats, previously showing increased fibroblast activity (Brown, Ritzenthaler et al. 2007). The administration of OTC in ovalbumin-induced murine models of asthma reduced the increased levels of ROS and airway inflammation (Lee, Kim et al. 2006). In addition, OTC administration appears to be capable of reducing VEGF concentrations, thereby reducing vascular permeability (Lee, Park et al. 2005).

The administration of OTC may increase the concentrations of the anti-oxidant glutathione. This will reduce activation of pro-inflammatory pathways and consequently decrease scar tissue formation and promote the healthy regeneration of damaged tissue. Its potential role in vascular permeability in damaged tissues and normalization of fibroblast activity may also contribute to its role in improved healing following spinal laminectomy.

2.0 Hypothesis and Research Objectives

Excessive inflammation promotes fibroblast mediated extra cellular matrix deposition, resulting in increased scar formation (Monaco and Lawrence 2003). Both quercetin and OTC are excellent anti-oxidants and anti-inflammatories that have been
previously shown to improve wound healing following injury (Phan, Sun et al. 2003; Lee, Kim et al. 2006). Therefore, the hypothesis being tested is that these agents will reduce inflammation, resulting in reduced scar tissue formation.

Investigations were conducted to determine if treatments with quercetin or OTC had a beneficial effect on the morphology of the healing tissues. In addition, the numbers of various inflammatory cells within the scar tissue were investigated in the developing scar tissues of the experimental treatment and the saline control groups. Specifically, I investigated the numbers of activated macrophages, mast cells as well as CD4 and CD8 T lymphocytes.

3.0 Materials & Methods

3.1 Preparation of Rats for Surgery

All protocols have been approved by the Animal Resources Center at the University of Saskatchewan and are in accordance with the Canadian Council on Animal Care.

Male Wistar rats (Animal Resources Centre, Saskatoon, SK, Canada and Charles River, Laval, PQ, Canada) (~250g) were weighed and anesthetized with 5% halothane (MTC Pharmaceuticals, Cambridge, ON) in oxygen. The dorsal skin was shaved and cleaned with hibitane (chlorhexidine gluconate) and 70% ethanol where the incision was made. At this time the rats were also given 0.05 mg/kg of buprenorphine (CDMV, St. Hyacinthe, Quebec) subcutaneously for pain management. With maintained anesthesia at 1.5-2% halothane, a spinal laminectomy was then performed.

3.2 Surgical Protocol for Spinal Laminectomy

An incision was made in the skin using a scalpel: scissors were then used to spread the muscular tissue away from the vertebral column on both sides without cutting, but tearing the muscle and ligaments from the spinal column. The muscle tissue was held back using chest spreaders. A blunt screwdriver was then used to spread the muscle
further laterally for unobstructed access to the spinal column. The use of the blunt
dissection reduces tissue damage and bleeding throughout the procedure. The laminae
of lumbar vertebrae L1 through L3 were removed in pieces using rongeurs. Figure 1
shows the location of the spinal laminectomy and where the laminae were initially
removed. Once the laminae were cut away the remaining portions of the vertebrae were
removed. Following this removal the muscle tissue was sewn together using 0-3 vicryl
absorbable sutures and the skin incision was then stapled closed. The rat then received
2 ml of lactated ringers solution (Abbott, Saint Laurent, Qu.) subcutaneously to restore
lost bodily fluids. To alleviate pain buprenorphine was administered every 12 hours
following surgery for 72 hours at decreasing doses (0.04 mg/kg, 0.03 mg/kg, 0.02
mg/kg, and 0.01 mg/kg). Suture staples were removed seven days following surgery.
Food and water was available ad libitum throughout the animals care.

3.3 Perfusion and Harvesting of Tissues

A total of fifty four rats were divided into three groups of eighteen to be killed at three
days, twenty one days, and sixty three days following surgery. These three groups of
eighteen were further divided into groups of six with each group treated with saline,
quercetin or L-2-oxothiazolidine-4-carboxylate (OTC). The quercetin-treated rats
received 0.025 mmol/kg of quercetin suspended in normal saline (Abbott, Saint Laurent,
Qu) by intra-peritoneal injection one hour after surgery and then every 12 hours for 10
days. The OTC-treated animals received 1 mmol/kg of OTC dissolved in normal saline,
and the saline-treated animals received saline 30 minutes post-surgery and then every
12 hours for 5 days via intra-peritoneal injection. Rats were anesthetized using 5%
halothane and an incision was then made opening up the abdominal cavity. Two
additional incisions were made laterally through the rib cage and the diaphragm was
excised from the body cavity. A blunt end needle was used to puncture the left ventricle
of the heart and held in place with a hemostat once it reached the aorta. The right
atrium was then cut to allow the release of blood. At this time a cold saline drip was
begun until the fluid ran clear. Fixatives were not used at this time as they would
Figure 1  Full rat skeleton with red arrow pointing to region of spinal column spinal laminectomy was performed (L1-L3) (a), and a human vertebrae indicating location of laminectomy (black dotted lines) and the portion of the spinal column removed (green arrow) (b).
interfere with data analysis of these tissues by research collaborators in Winnipeg, Manitoba. All animal from each group were included in the analysis.

The region of the back containing the desired scar tissue at L1 through L3 was then removed. The scar tissue was isolated with use of fine scissors and a scalpel blade, with bone fragments removed with the rongeurs. The tissue was then placed in a mold containing O.C.T. Compound (VWR Scientific, West Chester, PA) and dipped in isopentane cooled with liquid nitrogen in order to flash freeze the tissue. Frozen sections were cut at 10 µm.

Each block was serially sectioned transversely through the scar when the full scar became visible in the tissue block (Figure 2). The sections were placed on forty slides, two sections per slide. The tenth, twentieth, thirtieth, and fortieth slides were MirrIR slides (Kevley Technologies, Chesterland, OH) and used for infrared spectra analysis by research collaborators. The remaining slides were precleaned superfrost plus micro slides (VWR Scientific, West Chester, PA). Tissue sections on slides were stored at -80°C until stained.

3.4 Histology

3.4.1 Hematoxylin & Eosin

Hematoxylin & Eosin (H&E) staining was performed according to Humason (1979) as used for Delafield’s (or Harris’s) Hematoxylin, with the following modifications. Frozen sections were removed from the freezer and held at room temperature for forty-five minutes to one hour. They were then fixed in methanol at room temperature for thirty minutes. Dehydration consists of sequentially rising concentrations of alcohol solutions, one minute in 50:50 xylene:absolute alcohol, followed by sequentially rising concentrations of xylene. Erlich’s hematoxylin was used in lieu of Harris hematoxylin as it produced better staining results. Following running tap water, slides were quickly dipped in acid alcohol (0.5% HCl in 95% ethanol) and put back under running tap water.
Figure 2  Diagram of a rat showing the orientation of the sectioned tissues. Blue line is parallel to cuts made in sectioning. Blue box shows orientation of tissues on slides following sectioning. Black arrow indicates direction of sectioning: rostral to caudal.
Slides were then immersed in saturated lithium carbonate (1.36g/100mL) for 15 seconds and returned to running tap water to intensify the color of the stain. Eosin staining was then followed by the dehydration steps that included increasing concentrations of ethanol followed by 50:50 ethanol:xylene and then 100% xylene steps.

### 3.4.2 Toluidine Blue

Toluidine Blue staining was performed as outlined in Humason (1979) with the following modifications. Frozen sections were removed from the freezer and remained at room temperature for forty-five minutes to one hour. The sections were then fixed in room temperature methanol for thirty minutes. The toluidine blue stain used adjusted to have a pH of 3.0 in order to amplify metachromatic staining.

### 3.4.3 Analysis of Histological Materials

All slides were examined using a Leica DRMD microscope and photographed using a Nikon Cool Pix digital camera mounted to the microscope. The area and widths of the scar tissue was calculated using sections of the entire slide as scanned with a Nikon Super Coolscan 5000 at 4000 pixels per inch (ppi) at 100%. Scale bars were scanned to determine exact magnification of tissues. ImageJ 1.37G software was then used to measure the areas and distances as marked using the tracing and line measurement tools respectively.

### 3.5 Immunocytochemistry

#### 3.5.1 DAB Immunocytochemistry

The following antibodies obtained from AbD Serotec were used to detect activated macrophages, CD8+ T-lymphocytes, and CD4+ T-lymphocytes respectively: ED1, OX8, and W3/25. Detection was via the Vector laboratories Vectastain ABC kit (PK-6200) with the peroxidase substrate kit DAB (SK-4100). Controls were run to ensure no
background staining would occur by initially performing protocols with slides having no primary antibody, no secondary antibody, and both primary and secondary antibodies. The OX8, W3/25, and ED1 antibodies were proven to be specific at marking the CD8 T lymphocytes, CD4 T lymphocytes, and activated macrophages by previous studies (Bukovsky, A., et al. 1984; Barclay, A.N. 1981; Damoiseaux, J.G.M.C. et. al. 1994 ). The sections were stained as described in the Vector laboratories Vectastain ABC kit (PK-6200) instruction pamphlet with the following modifications. Sections were fixed with 4% paraformaldehyde in 0.03M PBS for 1 hour at room temperature. 0.03M PBS was the buffer solution used throughout the protocol for four 5 minute washes rather than the single 5 minute wash suggested. Endogenous peroxidase was inactivated by incubation in 0.1% H$_2$O$_2$ in methanol for one hour at room temperature. Incubation in blocking solution comprised of 10% horse serum, 0.03M phosphate buffered saline (PBS) and 1% Triton X was performed overnight at 4°C. Incubation with primary antibody was also performed overnight at 4°C. No counterstain was used on these slides. Dehydration steps were followed by mounting with Entellen mounting medium (EM Science) and a coverslip. Positive results were visualized as a dark brownish stain on the specific cell types investigated.

3.5.1.1 Cell Counting Procedures

Entire sections were digitally scanned with a Nikon Super Coolscan 5000 at 4000 pixels per inch (ppi) at 100%. The contrast of the scanned images was then increased using Adobe Photoshop 6.0. Images were then imported into ImageJ 1.37G software and cell positive profiles counted using the Color Deconvoluter in the Region of Interest (ROI) function to isolate the positively reacting cell types followed by the Analyze Particles function. Each section was counted 3 times. The scar tissue on each section was traced and the areas measured with ImageJ software. Artifactual spaces within the scar tissue were measured and subtracted. Areas within the scar tissue considered to be artifact were those spaces created by the sectioning process resulting in tears in the tissues when placed on the slides. Number of cells/mm$^2$ of scar tissue were calculated.
3.5.2 Propidium Iodide Staining

Propidium Iodide is a fluorescent DNA-binding stain that allows the recognition of the total number of cells within a section of tissue. A propidium iodide (PI) (Sigma 25535-16-4) stain was made by adding 1 μL of a PI stock solution (4.6 mg/mL) to 1 mL of a 1:1 mixture of glycerol and PBS. Frozen sections were removed from the freezer and remained at room temperature for forty five minutes to one hour. The section was covered with stain solution, a cover slip was applied and sealed with clear nail polish.

3.5.2.1 Cell Counting Procedures

The fluorescence of the PI stain was detected using a Nikon Y-FL Epi-fluorescence attachment with a tetramethylrhodamine isothiocyanate (TRITC) filter (excitation wavelength 547 nm; emission wavelength 572 nm). Images were taken using the 20X magnification lens. Three representative images per slide were captured. ImageJ 1.37G software was used to increase the contrast of these pictures by 40 points thereby more clearly isolating the positively staining nuclei. The nuclei were then quantified using the Cell Counter Plug-in.

3.6 Western Blot Analysis

Additional surgeries were performed as above with the same numbers for the use of western analysis, with the exception that only the 3 and 21 day time frames were examined. Groups of 6 were maintained with the exception of the saline and OTC treatments at 21 days, which had groups of 5. Following harvest of the scar tissue, tissue lysis was accomplished by the immediate addition of the tissue to a 1X Tris Buffered Saline (TBS) solution containing a phosphatase and protease inhibitor cocktail (Sigma) and homogenization with a Polytron homogenizer. Equal volumes of 2X SDS and urea buffer were added before being sonicated with a VibraCell™ (Sonic & Materials Inc., Danbury, CT, USA). Sonication was performed at a medium to high pulse range
between 30 to 45 seconds on ice. The tissue homogenates were then centrifuged in order to remove any undissolved protein before freezing them at -40ºC for future use.

Protein concentrations were estimated using the methods described in Minamide et. al. (1990) with the following modifications. The standard curve was determined using a dilution of a 1:3 mixture of 5 mg/mL bovine serum albumin (BSA) and 5 mg/ml γ-globulin. One sample from each rat was run in an individual lane with 10µg of loaded protein per lane. Western blots were performed as in Ausubel et al. (1988) with the following modifications. Electrophoresis was conducted on a 5%/12.5% split acrylamide gel that was self-poured and run between 75 volts and 85 volts to ensure even protein migration through the stacking gel until the protein samples reached the resolving gel. At this time the voltage was increased to 110 volts to 120 volts for about two hours as proteins migrated through the resolving gels, and until the loading dye had migrated to the bottom of the gel. The gels were then removed and placed in Petri dishes full of cold loading buffer for 20 minutes to equilibrate the gels. Proteins were transferred to nitrocellulose from the gel at 100 volts for 1 hour. Primary mouse antibody for vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Inc.) was diluted 1:1000 in 2% milk powder dissolved in phosphate buffered saline tween 20 (PBST) (0.8 g of milk powder in 42.5 mL of PBST) overnight at 4ºC. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as internal loading control reference. A mouse antibody against GAPDH was obtained from Chemicon International. A peroxidase-labelled anti-mouse secondary antibody (Sigma Immuno Chemicals A-1806) was diluted 1:10 000 in a 2% solution of milk powder dissolved in PBST (0.8 g in 42.5 mL) for 1 hour at room temperature. Secondary antibody was detected using a chemiluminescence reaction using Western Lightning Chemiluminescence Reagent Plus from Perkin Elmer (NEL105) and exposed to x-ray film. All films were scanned into digital format and analyzed by densometric analysis using ImageJ computer software. A clear spot on the blot was used as a blank before measuring the intensity of the bands. Both experimental and GAPDH control band intensities were measured so that the VEGF band intensities could be divided by the GAPDH intensities to determine their ratio.
3.7 Statistics

Statistical analysis of all data was performed with GraphPad Prism 4. The variable distribution of the data points obtained made it necessary to perform data transformations before statistical analyses were performed. Areas and distances were log transformed. Cell count data were transformed with a square root transformation as it is the standard transformation used when data involves counts (McDonald, 2008). Cell densities as a percentage of the total cell density were arcsine transformed as it is the standard transformation used for ratio and percentage data. Data were compared using a two way analysis of variance (ANOVA) test with a Bonferroni’s post hoc test comparing all columns of data. Statistical significance was considered if the P value was less than 0.05.

4.0 Results

4.1 Qualitative Observational Results

Several observations were made concerning differences between the experimental treatment groups and the saline control groups during the care and maintenance of the rats in the 21 day and 63 day post surgical groups, as well as in a separate study with rats sacrificed at 7 months following laminectomy. Differences amongst the treatment groups include the extent of scar tissue formed in the skin at the incision site and at the skin suture staple sites, hair regrowth over the surgery site, and the regrowth of bone at the laminectomy site.

As healing progressed it was observed that the incision line and corresponding round marks left behind by the skin suture staples were less obvious in the experimental treatment groups than those in the saline control groups (Figure 3). In the experimental treatment groups scarring from the incision site and staple suture sites were uncommon, and often non-existent. Both incision and staple suture sites were difficult to see on rats treated with OTC, requiring close inspection to determine the exact location of the laminectomy. These scars appeared to be fully healed and seldom exhibited a
Figure 3  Skin scars on recently shaved saline treated rats (a), quercetin treated rats (b), and on OTC treated rats (c) seven months following spinal laminectomy. Large arrows indicate the length of the laminectomy and smaller arrows indicate scar left behind by suture staples.
significant difference in pigmentation between the wound areas and the surrounding skin. Saline control groups typically had raised scars with a swollen appearance and very little skin pigment making them very obvious along both the incision site and the sites of the suture staples.

In addition, it was observed that experimental treatment groups had an increased rate of hair growth over the laminectomy site as compared to the saline control groups. Interestingly, the hair growth in the OTC and quercetin treated groups occurred most prominently on the healing laminectomy site and incision line, but was much slower in the areas shaved that were furthest from the healing laminectomy site. It appeared that the hair growth occurred initially at the laminectomy site and over time spread out to the areas furthest from it. Hair growth following surgery in the saline treated controls was slower to occur following surgery and appeared sporadic and patchy with no consistent pattern in relation to the laminectomy site.

It was also noted in the 63 day post surgery and 7 month post surgery animals that bone regrowth at the site of laminectomy had occurred faster in the OTC treatment group than the saline control group. When harvesting the scar tissue for analyses the fill-in scar tissue smaller in size and the adjacent bone was denser and more difficult to remove in the experimental groups, especially in the OTC-treated group. Less of bone regrowth was typically seen in the saline control groups, and the bone was much softer and easier to remove at the time of tissue harvesting.

4.2 Histology

4.2.1 Hematoxylin and Eosin Stained Scar Tissues

At 3 days post surgery very few differences were seen between treatment groups. There is a general lack of detectable organization in the tissues. However, slight differences in the fibrillar components of the matrix were present. The quercetin slides have a more mottled appearance with some extra cellular matrix apparent in the tissues; the OTC treated samples begin to show sporadic fibrillar patches of collagen fibrils. The saline treated samples appear more cellular in nature with a lack of fibrillar
appearance. Figure 4 shows typical examples of these slight differences in the 3 day post surgery tissues.

By 21 days after surgery there were clear differences between the experimental groups and the controls. Both experimental treatment groups had more fibrillar and more organized areas of scar tissue than the saline treated control. Although fibrillar patches are visible in the saline treated samples, they are much less prominent and less frequent than in the quercetin and OTC treated samples. Figure 5 shows typical examples of these differences observed in the 21 day post surgery tissues.

When observing the complete sections of the 21 day post-surgical tissues it becomes apparent that differences also appear to exist at this level of tissue repair. The quercetin-treated samples typically show a smaller area or lateral width taken up by scar tissue within the entire section and often the scar tissue is beginning to clearly connect or adhere to the surrounding muscle. Similar observations were seen in the OTC treatment group. Saline-treated tissues maintain a larger expanse of disorganized scar tissue with little of it beginning to clearly adhere to the surrounding tissues. Therefore the tissue sections from the experimental tissues tend to have a significant amount of muscular tissues surrounding and adhering to the scar tissue, whereas the saline control tissues appear to be largely composed of scar tissue itself with less muscular surrounding tissues. In the experimental treatment groups the scar tissues also appear to have a smaller medio-lateral dimension than in the saline controls. Figure 6 shows representative full section scans of the healing tissues at 21 days following laminectomy. Measurements of the areas and scar tissue widths are discussed in section 4.5.

4.2.2 Toluidine Blue Stained Scar Tissues

Staining is said to be metachromatic when a single stain produces more than one colour according to the molecule stained. This is accomplished due to the nature of the toluidine blue stain, which organizes itself in an orderly manner on regular polyanionic surfaces. Glycosaminoglycans (GAGs), which are commonly found in close relation to collagens and other extracellular matrix (ECM) components, are rich in these polyanionic surfaces. By using a toluidine blue stain at a low pH, metachromatic
Figure 4  H&E stained scar tissue sections 3 days following laminectomy in saline treated animals (a), in quercetin treated animals (b), and in OTC treated animals (c). Scale bars = 50 micrometers.
Figure 5  H&E stained scar tissue sections 21 days following laminectomy in saline treated animals (a), in quercetin treated animals (b), and in OTC treated animals (c). Scale bars = 50 micrometers.
Figure 6  H&E stained full section scar tissue scans 21 days following laminectomy in saline treated animals (a), in quercetin treated animals (b), and in OTC treated animals (c). Arrows indicate margins of scar tissue next to the surrounding muscular tissues. Scale bars = 100 micrometers.
staining is enhanced. The resultant patches of pinkish hue within the blue stained tissues indicate regions more concentrated in collagen due to the associated glycosaminoglycans (Figure 7). Therefore this metachromatic staining indicates areas rich in scar tissue. Very little metachromasia was observed in the surrounding muscular tissue. The metachromatic staining observed within the scar tissue aided the research by colleagues in Winnipeg who used Fourier Transform Infrared (FTIR) spectroscopy to investigate the nature of collagen and its associated molecules within the healing tissues (Wiens, Rak, et al. 2007). By observing the areas with higher levels of metachromatic staining, they were better able to select regions within the scar tissue to investigate. These metachromatically stained areas were found throughout the saline control tissues, but appeared more concentrated in areas of a more fibrillar nature in the experimentally treated tissues.

Metachromasia of granules within mast cells enabled recognition of mast cells. Mast cells were analyzed both within the scar tissue itself and in the surrounding muscular tissues. In all sections examined, the mast cells commonly appear to cluster together in groups at the edges of the scar tissue. It was also observed that areas with a more fibrillar appearance in the central region of the scar tissue have mast cell clusters in close proximity. Only rarely were mast cells observed alone in the unorganized scar tissue. Those mast cells occurring in the surrounding muscular tissues were scattered in an apparent random manner throughout the tissue with no visible cell clustering. These patterns are apparent in all treatment groups at both 3 days and 21 days post surgery. The distribution of mast cells seemed to be unaffected by treatment.

4.3 Cell Content in the Scar Tissue

4.3.1 Total Cell Content

Figure 8 shows the average total cell density as measured in three selected areas within the scar tissues. Statistical analysis with the square root transformed data reveals that observed changes are not statistically significant, with the exception of the
Figure 7  Toluidine Blue stained scar tissue sections 3 days following laminectomy in saline treated animals (a), in quercetin treated animals (b), and in OTC treated animals (c). The pinkish violet hue represents glycosaminoglycan distribution. Scale bars = 50 micrometers.
Figure 8  Total cell density (cells/mm² scar tissue). Graphs represent the mean +/- SEM of the data. Statistical analysis performed using transformed data values.
changes in cell numbers over time (Interaction: P = 0.9166; Time: P=0.0029; Treatment: P=0.9831; 2 way ANOVA). This means that there are significant decreases in cell numbers/unit area from the 3 day to 63 day time point when considering all treatment groups.

4.3.2 Mast Cells

4.3.2.1 Mast Cells within the Scar Tissue

Total numbers of mast cells quantified in the scar tissue itself are presented in Figure 9a. A 2 way ANOVA of the square root transformed data indicates the slight fluctuations observed in mast cell numbers are not statistically significant (Interaction: P = 0.7909; Time: P=0.5471; Treatment: P=0.7435).

Mast cell density within the scar tissue was determined using the scar tissue area measured in the slides stained to determine activated macrophage concentrations as a basis for normalization of the numbers. This particular slide was chosen as it is the slide directly beside the slides stained with toluidine blue in the serially sectioned slides. The mast cell density within the scar tissue indicates no statistical significance amongst treatment groups or over time (Interaction: P = 0.6494; Time: P=0.6226; Treatment: P=0.9699). (Figure 9b)

The number of mast cells as a percentage of the total cells within the scar tissue is illustrated in Figure 9c. Again there were no statistically significant differences amongst treatment groups nor over time (Interaction: P = 0.8133; Time: P=0.0904; Treatment: P=0.6338; two way ANOVA).

4.3.2.2 Mast Cells within the Muscular Tissues Surrounding the Scar Tissue

Changes in the mast cell numbers within the muscular tissues surrounding the scar tissue (Figure 10a) were square root transformed and a 2 way ANOVA test found them to be statistically insignificant when considering the treatment at all time frames and the interaction between the treatment and time frames (Interaction: P =0.2770;
Figure 9  Mast cell numbers in scar tissue (a), mast cell density (cells/mm² scar tissue) (b), mast cell density as a percentage of total cell density (c). Graphs represent the mean +/- SEM of the data. Statistical analysis performed using transformed data values.
Figure 10 Mast cell numbers in the muscular tissues surrounding the scar tissue (a), mast cell density in the muscular tissues surrounding the scar tissue (cells/mm² scar tissue) (b), mast cell density as a percentage of total cell density (c). Graphs represent the mean +/- SEM of the data. Statistical analysis performed using transformed data values.
Treatment: P = 0.9188), however the changes in cell numbers over time is significant (Time: P=0.0242).

To determine whether there is a relationship between the size of the scar tissue and numbers of mast cells in the surrounding muscle, the mast cell numbers within the muscular tissues surrounding the scar tissue were related to scar tissue area. This will be referred to as muscle scar mast cell density. These data are illustrated in Figure 10b. Analysis of the square root transformed data indicates that no statistical significance exists between the interaction of the time and treatment, nor with the treatment alone (Interaction: P = 0.3267; Treatment: P=0.7922). It was again shown that the increases in cell numbers over time regardless of the treatment was significant (Time: P=0.0264).

The mast cell percentage of the total cells, in figure 10c, also indicates a statistically significant increase in cell numbers over time (Time: P=0.0010), however no significance is indicated for the interaction of time and treatment, or the treatment alone (Interaction: P=0.3031; Treatment: P=0.9220).

It is likely that these instances of statistical significance are informing us of the increases in cell density percentage seen between all 3 days samples and all 21 day samples.

### 4.3.3 Activated Macrophages

As shown in Figure 11a, macrophage cell numbers are showing similar trends as seen with the mast cells. A 2 way ANOVA indicates that the interaction of the treatment and time frames do not show statistical significance (Interaction: P=0.5345). The treatment alone also shows no significance (Treatment: P=0.2504). However, there is statistical significance in the decrease of macrophages over time (Time: P<0.001). A Bonferroni’s post hoc test shows that there are significant decreases in cell numbers over time from 3 days post surgery to 21 days post surgery within the quercetin treated groups (P<0.05). The decrease in macrophage numbers between the 3 and 63 day time points show significance among all the treatment groups with the saline-treated control having the least significance (Saline: P<0.05; Quercetin: P<0.001; OTC: P<0.001). And
Figure 11 Macrophage cell numbers in scar tissue (a), macrophage cell density (cells/mm² scar tissue) (b), and macrophage numbers as a percentage of total cell numbers (c). Graphs represent the mean +/- SEM of the data. Statistical analysis performed using transformed data values.
finally the saline-treated control group shows significant decreases in macrophages between the 21 day and 63 day post surgery time points (Saline: P<0.05).

Statistical analysis of the macrophage cell density within the scar tissue, in Figure 11b, indicates statistically significant decreases over time, as well as throughout the treatment groups without consideration of the time points (Time: P=0.0058; Treatment: P=0.0358), however no significance is seen in the interaction between the two (Interaction: P=0.6552). The Bonferroni’s post hoc test indicates the saline treated control has significant decreases in macrophage density between the 3 day and 63 day time points (P < 0.05).

Macrophages as a percentage of total cells was also calculated (Figure 11c) Surprisingly, a 2 way ANOVA indicates no significance over time and amongst treatment groups with these data (Interaction: P=0.8208; Time: P=0.2910; Treatment: P=0.1177).

**4.3.4 CD4 T Lymphocytes**

CD4 T lymphocytes were found to have statistically significant decreases (Figure 12a) in the interaction of their numbers within the treatment groups and the time points investigated (Interaction: P=0.0399), as well as over time independent of the treatment (Time: P<0.0001). There was so significant effect of treatment on CD4 T cell numbers (Treatment: P=0.8575). The Bonferroni’s post hoc test found a number of significant differences in cell numbers. Quercetin treatment was associated with a significant reduction of CD4 T cells between the 3 day and 21 day time points (P<0.05). Between the 3 day and 63 day post surgery time points both the OTC (P<0.01) and the quercetin (P<0.001) treated groups showed significant reductions. And finally the 21 day and 63 day time points indicated significant decreases in CD4 cells in the OTC (P<0.05) and saline (P<0.05) treated groups.

Figure 12b show the CD4 T lymphocyte cell density within the scar tissue. A 2 way ANOVA of the square root transformed data indicated no statistical differences in cell density between the treatment groups over time (Interaction: P=0.3724; Time: P=0.0657; Treatment: P=0.5745).
Figure 12 CD4 T Lymphocyte cell numbers in scar tissue (a), CD4 T Lymphocyte cell density (cells/mm² scar tissue) (b), and CD4 T Lymphocyte cell numbers as a percentage of total cell numbers (c). Graphs represent the mean +/- SEM of the data. Statistical analysis performed using transformed data values.
CD4 T lymphocytes as a percentage of total cells, indicated in Figure 12c, also indicated no statistically significant changes over time or amongst treatment groups. The 2 way ANOVA indicated that the interaction of time and treatment together had a P=0.4023, the time independent of treatment had a P=0.1057, and the treatment alone had a P=0.6885.

Despite the lack of statistical significance in the CD4 T lymphocyte density and percentages, there does seem to be a trend observed for decreases in the later time points.

**4.3.5 CD8 T Lymphocytes**

Figure 13a shows the number of CD8 T lymphocytes quantified in the scar tissue. Statistical analysis showed no statistically significant changes in these cell numbers over time or amongst treatment groups (Interaction: P=0.7320; Time: P=0.1021; Treatment: P=0.2008).

The CD8 T lymphocyte densities were also found to have no statistically significant changes when analyzed by 2 way ANOVA (Interaction: P=0.7554; Time: P=0.2472; Treatment: P=0.2982). These data are illustrated in Figure 13b.

CD8 T lymphocytes as a percentage of the total cell numbers indicates a significant difference in the proportion of cells that are CD8 over time (Time: P=0.0498), but does not show any significance with treatment alone (Treatment: P=0.6177) or the interaction of the treatment over time (Interaction: P=0.7236). The trend observed in this data (Figure 13c) is an unexpected increase in CD8 T lymphocytes in the later time points.

**4.4 T Lymphocyte Ratios with the Scar Tissue**

The ratios of CD4 to CD8 T lymphocyte cell numbers in the two experimental groups (seen in Figure 14), appears to be higher at the initial 3 day time point and compared to the 21 and 63 day time points. Two way ANOVA indicated that these
Figure 13 CD8 T Lymphocyte cell numbers in scar tissue (a), CD8 T Lymphocyte cell density (cells/mm² scar tissue) (b), and CD8 T Lymphocyte cell numbers as a percentage of total cell numbers (c). Graphs represent the mean +/- SEM of the data. Statistical analysis performed using transformed data values.
Figure 14  CD4:CD8 T Lymphocyte cell number ratios in the scar tissue. Graphs represent the mean +/- SEM of the data. Statistical analysis performed using transformed data values.
observed trends are not statistically significant (Interaction: P=0.3381; Time: P=0.0878; Treatment: P=0.6513).

4.5 The Area and Widths of Scar Tissue

Figure 15 shows the areas of scar tissue. The decreases in scar tissue area over the three time points investigated indicate significant reductions over time (Time: P<0.0001), however these changes are independent of the treatments (Treatment: P=0.4999; Interaction: P=0.1145). The Bonferroni’s post hoc test singles out differences found only between the treatment groups, and no significant reductions seen in the saline treated controls. The differences observed between the 3 day and 63 day time points for both the OTC and quercetin treated groups indicated P<0.001. Again, the OTC and quercetin treated groups both indicated significant reductions during the 21 day and 63 day healing periods (OTC: P<0.001; Quercetin: P<0.05)

As seen in previously in Figure 4, there appears to be differences in the lateral width of scar tissue occupying the peridural space following laminectomy. These distances were measured at the dorsal (top), middle and ventral (bottom) aspects of the scar tissue between muscle layers (Figure 16 a, b, and c). In addition the average of all these width values were taken and calculated as the average width for the peridural scar tissue. (Figure 16d) As expected, the widths in each instance decreased over time from the 3 day to the 63 day time points.

The measured widths at the top of the infiltrating peridural scar tissue indicate statistical significance when time and treatment are considered independently, but do not show significant differences when considering the interaction of the two (Interaction: P=0.4834; Time: P<0.0001; Treatment: P=0.0131). The Bonferroni’s post hoc test indicates significant reductions between the 3 day and 21 day time points in all treatment groups (OTC: P<0.05; Quercetin: P<0.01; Saline: P<0.01).

The widths taken at approximately the middle of the scar show significant differences in the width over time, but do not appear to show significant differences with respect to the treatments (Interaction: P=0.7048; Time: P<0.0001; Treatment: P=0.0845). The Bonferroni’s post hoc test indicates significant reductions in width
Figure 15 Scar tissue areas (mm$^2$). Graph represent the mean +/- SEM of the data. Statistical analysis performed using transformed data values.
Figure 16  Scar tissue width measured at the top of scar (a), scar tissue width measured in the middle of scar (b), scar tissue width measured at the bottom of scar (c), and the average width of the scar (d). Graphs represent the mean +/- SEM of the data. Statistical analysis was performed using transformed data values.
between the 3 day and 63 day post surgery time points for all treatment groups (OTC: P<0.01; Quercetin: P<0.05; Saline: P<0.001). Despite the lack of significance, the general trend observed is decreased widths in the experimental treatment groups.

The widths at the bottom of the peridural space close to the site of laminectomy indicate statistically significant results in all aspects tested. The interaction of the treatment and time indicate a P=0.0216. Time independent of treatment shows reductions in width equating to a P<0.001, and the treatment alone shows a P=0.0131. The OTC treated group (P<0.0001), quercetin treated group (P<0.001), and the saline treated controls (P<0.01) all indicate significant reductions in scar width closest to the laminectomy site. Again, a trend in reduced widths can be seen in the experimental treatment groups, particularly in OTC treated group.

The average widths of the peridural scar are lower in the experimental treatment groups in each of the time points investigated (Figure 16d). A 2 way ANOVA of this log transformed data indicates statistical significance in the reduction of scar tissue width when considering the time and treatment parameters independently, but not with the interaction of the two (Interaction: P=0.6927; Time: P<0.0001; Treatment: P=0.0074). All groups indicate a significant change with post hoc testing between the 3 day and 63 day post surgery time points (OTC: P<0.001; Quercetin: P<0.01; Saline: P<0.001). Both the OTC and quercetin treated experimental treatment groups tend to have reduced average scar tissue widths at all time points, although no significance is indicated.

4.6 Western Blot Analysis

4.6.1 VEGF Protein Concentration

VEGF protein levels, when normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are shown in Figure 17. Two way ANOVA analysis with log transformed data suggests fluctuations in VEGF concentrations when normalized to GAPDH are not statistically significant when considering the treatment as a parameter (Interaction: P = 0.4780; Treatment: P=0.8822). There is an observed change in VEGF when examining the changes in the protein over time independent of the other
Figure 17 VEGF protein levels normalized to GAPDH in the scar tissue. Graphs represent the mean +/- SEM of the data. Statistical analysis performed using transformed data values.
parameters (Time: P=0.0311). It appears that VEGF protein concentrations increase between the 3 day and 21 day post surgery time points, regardless of treatment.

5.0 Discussion

5.1 Predicted Results Following Literature Review

After reviewing the literature on studies investigating wound healing and scar tissue formation, it was expected that treatment with OTC and quercetin following injury would result in a significant reduction in the inflammatory response thereby ameliorating scar tissue formation. The process of wound healing and scar tissue formation has been shown to follow a common and predictable path of events, and commonly theories on scarring are tested with the premise that the interruption or amelioration of specific inflammatory events may lead to better healing. Fetal tissues and oral mucosa heal faster than other tissues with little or no scar tissue formation (Samuels and Tan 1999; Szpaderska, Zuckerman et al. 2003; Fraser, Cuttle et al. 2005; Szpaderska, Walsh et al. 2005; Kishi, Ohyama et al. 2006). The different mechanisms of inflammation found in fetal tissues play a profound role in this regeneration of healthy skin following injury. For instance, fetal tissues exhibit less angiogenesis, a marked reduction in inflammatory cells and growth factors infiltrating the wound, and differences in extracellular matrix proteins laid down (Adolph, DiSanto et al. 1993; Samuels and Tan 1999; Szpaderska, Walsh et al. 2005). Frantz et al discovered that adult-like healing can be induced in the normally scarless healing fetal tissues by encouraging an immune inflammatory response similar to that seen in adults from a bacterial infection (Frantz, Bettinger et al. 1993). They found that increasing the influx of neutrophils, macrophages, and collagen deposition in the fetal tissues resulted in scar tissue formation like that seen in adults. The bulk of the literature on the prevention of scar tissue formation attempts to determine ways in which to mimic these characteristics fetal repair in normal adult tissues to incur a more beneficial healing process, through the elimination or decrease of certain aspects of inflammation and extracellular matrix deposition.
The large majority of the studies focus on the mechanisms of wound healing and the resultant scar tissue formation in cutaneous tissues. Fetal wounds having the benefit of scarless healing is well known, but even fetal scar studies focus on the skin and its specific healing processes. Additional studies on fetal tissues have found injury to tissues other than the cutaneous tissues will result in a wound healing process resulting in a scar. Injury incurred in the same gestational period of time in which cutaneous scarless healing occurs resulted in scar tissue formation and a lack of muscle regeneration in the fetal diaphragm (Longaker, Whitby et al. 1991), as well as scar tissue formation following spinal nerve injury (Lin, Posnick et al. 1994).

A great many studies have, however, investigated various methods of preventing scar tissue formation following spinal surgery such as preservation of the ligamentum flavum (Ozer, Oktenoglu et al. 2006) and placement of a barrier between the exposed spinal cord and the surrounding muscular tissue (Klopp, Simon et al. 2008). Treatment with various anti-inflammatory supplements and drugs have also been shown to improve inflammation and scar tissue formation (Shah, Foreman et al. 1995; Kempuraj, Madhappan et al. 2005). However, very few consider that the mechanisms of the healing process may follow a different set of events dependent on the tissue types and physiological location in question. An injury to the skin will involve only cell types and processes specific to the cutaneous tissues. Whereas, an injury such as a spinal laminectomy brings into play a plethora of cell types and healing processes not present in the skin, such as osteoclasts and osteoblasts involved in bone repair. With the laminectomy we injure the skin itself in order to make an incision, but we also tear muscle and tendon in order to expose the spinal column, and injure the bone itself during the laminectomy process. The process of bone healing is complex and elicits the help of many cell types not seen in cutaneous wound healing (Ashhurst 1990; Cornell and Lane 1992). The introduction of the healing processes of bone and the muscular tissues have now additionally been included. The inflammatory events occurring in wound healing within deeper tissues may be characterized by slightly different inflammatory and cellular events. Barbul et al discovered that T lymphocytes increase during the first week in both superficial and deep wounds, but the numbers start
declining in the superficial wounds by ten days following injury, but remain constant in the deeper wounds (Fishel, Barbul et al. 1987; Barbul 1988).

5.2 Qualitative Observations Indicate Scar Tissue Amelioration

Observations made during the healing time following the surgery indicate that scar tissue formation following the spinal laminectomy was improved in those experimental groups treated with the anti-inflammatory supplements. Skin scars left behind in the quercetin and OTC treated rats were less raised, demonstrated almost normal skin pigmentation, and scars were not obvious to casual observation. Those scars left behind in the saline treated animals were most often raised, with little pigmentation in comparison to the surrounding skin, making them easily observable. Researchers who conduct studies on cutaneous tissue in models comparable to keloid and hypertrophic scarring would view these as positive results and often would take the height and width measurements of the scars for a more quantitative analysis. Saray et al was able to determine the efficacy of intralesional jet injection of bleomycin on keloid and hypertrophic scars solely on the scar height measurements and patient feedback (Saray and Gulec 2005). Unfortunately, the scars observed here were not raised to the extremes as those in hypertrophic and keloid scarring, but were confirmed by touch when handling the rats.

Histological results indicate that the morphology of the wound is more favorable in the experimental treatment groups as compared to the saline control. At 21 days post surgery the quercetin and OTC treated tissues exhibit more frequent bundles of organized tissue and there are also more sites of attachment occurring between the scar tissue and the surrounding muscular tissues. In previous studies performed in our lab, it was observed that quercetin treated animals having undergone spinal cord injury experienced enhanced wound healing in the cutaneous incision site. These tissues also appeared more organized morphologically in regards to the skins tissue layers and accessory structures. Other studies investigating scar tissue formation have had similar positive morphological results, but failed to find any qualitative results to accompany them. One such study found that aceclofenac, a NSAID, issued following spinal
laminectomy was found to improve adherence of the dura to scar tissue and decrease
the number of fibroblasts, but no differences in the types of inflammatory cells were
found (Sandoval and Hernandez-Vaquero 2008).

5.3 Quantifiable Results Suggest No Change in Numbers of Inflammatory Cells
Due to the Administration of Quercetin and OTC

I had hypothesized that quercetin and OTC treatments would decrease
inflammation in the incipient scar tissue. To examine this I counted the number of
inflammatory cells. As will be discussed below the number of inflammatory cells does
not necessarily reflect the extent of inflammation and, thus, may not have been the ideal
means to assess the extent of inflammation.

Analysis of macrophages indicated a significant decline when comparing
differences in all groups between the 63 day and 3 day time frames, with the most
significant differences observed in the experimental treatment groups. The data suggest
that the number of macrophages may be increased in the treatment groups more so
than in the saline control groups at 3 days post surgery, and may maintain slightly higher
levels across the time frames. It does appear that the experimental treatment groups
have increased numbers of macrophages at nearly all time points, despite the lack of
statistical significance when analyzed one cannot ignore the trend.

Macrophages, although an important leukocyte with many tissue destructive
functions, has also been shown to have a number of tissue protective functions.
Upregulation of macrophages and T cells during the inflammatory response by the
administration of dendritic cells exposed to encephalitogenic or nonencephalitogenic
peptides improved the outcome of spinal cord injury (Hauben, E. et al. 2003) It has also
been shown that heme oxygenase (HO) plays a role in both the development and
resolution of the inflammatory response, and increases in HO in the later stages of
wound healing appear to be beneficial. Interestingly Kampfer et. al. found that the
primary source of HO in these healing cutaneous wounds was the infiltrating
macrophages (Kampfer et al. 2001). There have been instances in which increased
levels of macrophages were actually found to be beneficial to the wound healing
Following myocardial infarction, increases in macrophage-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor (G-CSF) improved healing despite the corresponding increase in macrophages (Misao, Takemura et al. 2006; Morimoto, Takahashi et al. 2007).

There was a significant reduction in CD4 T lymphocytes between the 3 day post surgery time point and the 63 day post surgery time point in both quercetin and OTC treated groups, however, no significant differences were seen when experimental groups were compared to saline controls. This point is emphasized with the lack of difference in cell numbers amongst the three groups at the final 63 days post surgery time point investigated. There were also no significant differences amongst the treatment groups or over time in the CD4:CD8 T lymphocyte ratios. Keloid scars exhibit higher T cell numbers than that of normal skin, with further investigation revealing that the keloid tissues have significantly higher CD4:CD8 T lymphocyte ratios (Boyce, Ciampolini et al. 2001). However, a reduction in total T lymphocytes can result in impaired wound healing. The specific roles each T cell subset plays is unknown, but a reduction in CD8 T lymphocytes can result in a more beneficial result, whereas a reduction in CD4 T lymphocytes appears to have no affect on the wound healing process (Barbul, Breslin et al. 1989). Therefore the key to having beneficial T lymphocyte levels within the healing wound may simply be to prevent excessive numbers from infiltrating the wound and maintaining a healthy balance of the T lymphocyte subsets, in lieu of drastically reducing or eliminating their numbers. Another factor to consider is that T lymphocytes have been shown to peak at approximately day seven following injury (Regan 1994), a time point not considered in this particular study. It is possible that significant differences may have been more obvious if tissues had been tested closer to seven days following injury.

VEGF is considered the major growth factor responsible for the induction and maintenance of vessel growth following tissue injury. No significant changes were seen in its protein levels amongst the experimental treatment groups. Despite the fact that reduced angiogenesis has been associated with beneficial wound healing within oral mucosa (Szpaderska, Walsh et al. 2005), it has also been shown that reductions in angiogenesis have little effect on the outcome of wound healing in mice (Bloch, Huggel
et al. 2000). Wilgus et al examined levels of VEGF in a fetal scarless wounding model and discovered that VEGF may play a more diverse role in wound healing than the process of angiogenesis alone (Wilgus, Ferreira et al. 2008), therefore decreases in VEGF may not be beneficial to scar formation. Perhaps again, the key to beneficial wound healing does not require a reduction in VEGF, but maintenance of a healthy in excess concentration.

Despite observational results indicating a beneficial morphological change in those experimentally treated tissues, quantitative analysis of inflammatory cell infiltration showed no apparent differences. Given the bulk of literature suggesting an excessive inflammatory reaction is the perpetrator leading to scarring following injury, this was quite surprising. Although not statistically significant, my results appear to indicate that treatment with quercetin and OTC actually increases the inflammatory cell infiltration into the wound site in the earlier stages of wound healing and then decrease them in the later stages of repair.

Inflammatory cells are certainly not the only aspect of inflammation affecting wound healing, so possibly these treatments affect some other event occurring in the response to injury. Many cytokines and growth factors involved in wound healing have been shown to affect scar formation following injury. For example, the absence of monocyte chemotactic protein -1 (MCP-1), a cytokine produced early in wound healing and thought to attract monocytes and macrophages to the injury site, resulted in delayed healing despite no reduction in the numbers of infiltrating macrophages (Low, Drugea et al. 2001). Transforming growth factor beta (TGF-β) has been shown to be heavily involved in the wound healing process, although each of its isoforms appears to have different consequences to resultant scar tissue formation. Neutralization of TGF-β1 and TGF-β2 concurrently reduced inflammation and extracellular matrix deposition in a healing wound, but did not result in a more beneficial scarring process. Alternatively, increases in TGF-β3 concentrations also improved the outcome of wound healing resulting in a reduction of scar tissue formation (Shah, Foreman et al. 1995).

The areas of scar tissue on the slides analyzed for macrophages were measured and found to have some statistically significant differences. The quercetin treated tissues and the OTC treated tissues at 63 days post surgery had significantly decreased
scar tissue areas than in that of their 3 day counterparts. The treatment groups may therefore have incurred a larger reduction in scar tissue than observed in the saline controls over time. In particular the OTC treated tissues appeared to have the greatest reduction in area over time than any other group. In addition both the 63 day OTC and quercetin treated tissues had a significant reduction in scar tissue formation over the 21 day saline treated tissues. Again, suggesting the experimental treatments may be altering the scar tissue contraction. However, at no individual time point was there a significant difference between the experimental treatment groups and the saline control group.

Although these statistically significant values in changes in scar tissue area are interesting there is no difference in the final area of scar tissue between the experimental treatment groups and the saline control tissues. While the treatments do appear to result in slight decreases in the area encompassed by scar tissue in the experimental treatment groups, this study did not show an overall significant decrease in the area of the scar infiltrating the peridural space at the 63 day time point.

In addition to the areas of scar tissue, the widths were measured at the top, middle, and bottom of the scar tissue existing between the surrounding muscular tissues. The width at the top, middle and bottom of the scar tissue was significantly reduced in all groups between 3 days and 63 days. Like the areas measured, the majority of significant changes take place between the 3 day groups and the experimental treatment groups at 63 days following surgery. Again, these results could possibly be indicating decreases in scar tissue formation over time.

The averages of these results indicate that the most significant reductions occurred in the widths of the scar tissue are observed between the 3 day saline control time point and the 63 day time points treated with OTC and quercetin. Both the OTC and saline treated tissues indicated significant reductions between the 3 and 63 day time points above that of the differences seen in the quercetin treated group.

As expected, there are significant reductions in the size of the scar between the 3 day and 63 day time points. The results indicate that we may be seeing a slightly more significant decrease in scar tissue area from the 3 day samples in the experimental treatment groups at the latest time points. As with the measured areas, although there
is no statistical significance, the experimental treatment groups tend to exhibit slightly lesser widths than that of the saline control tissues at most time points. Therefore treatment with OTC and quercetin following laminectomy may be encouraging more beneficial wound healing more so in terms of the morphology of the resultant scar than the size of the scar itself.

5.4 Possible Explanations for Results Deviating from those Predicted

The external appearance of the scar as well as the histology of the scar tissue indicated that our OTC and quercetin treatments have some therapeutic effects in the cutaneous aspect of the scar. However, the quantitative analyses of immune cell numbers and scar tissue areas did not show robust difference amongst the three treatment groups in the entire scar. It may be that these are not the appropriate parameters to assess the extent of inflammation or quality of scar tissue. The peridural scar, in hindsight, is a difficult model of scarring to quantify. The cutaneous hole punch model presents a more accessible and easily measured area of scar tissue for study, with the additional benefit of monitoring the scar itself over time without disruption or death to the animal.

Furthermore, every individual experiences different levels of scarring and inflammation based on diet, general health and heredity. Diets and the general health of the rats were monitored in these experiments, but genetics was not a factor considered. Studies have indicated that keloid scarring may be a hereditary trait in humans, having a higher incidence in certain races (Bayat, Arscott et al. 2003; Robles and Berg 2007). While these experiments used the same breed of rat, the male Wistar, it was not considered important at the time to ensure that the rats received were from the same genetic breeding line. The different genetic backgrounds of the rats used therefore may have played a role in the ultimate diversity of the data obtained.

Another factor to consider would be the treatment doses of quercetin and OTC administered. The doses of OTC and quercetin were decided based on previous dose response curves determined in studies in this lab investigating the effects of these treatments on spinal cord injury (Kamencic, Griebel et al. 2001; Schultke, Kendall et al. 52
2003). The doses administered produced positive results in terms of the morphological appearance of the scar, and can therefore be considered to be within the therapeutic range, it is possible that a higher dose would have had a greater impact on the inflammatory aspects of tissue repair. It was also noted during perfusion that the intraperitoneal cavity of the rats receiving quercetin treatment still harbored some of the injected supplement. Therefore, a different means of administering the same dosage of these agents may have also resulted in a greater impact on the influx of inflammatory cells during wound healing.

Using a model looking specifically at the effects of these treatments on the skin before investigating their effects on internal scarring may have also been beneficial. The skin punch model is a well known method of studying cutaneous wounding (Gerharz, Baranowsky et al. 2007), and may allow for more accurate measurements of the resultant scar tissue and the effects of the treatments. Unlike the peridural scar investigated here, the cutaneous scars are easier to assess due to their location and specific physiological features including their tissue layers and accessory structures such as hair follicles. The peridural scar has fewer physiological landmarks and its location requires surgical removal to analyze. It was necessary to remove bone in this particular study for use in FTIR spectroscopy and despite the utmost care in this process; it is possible that some tissue was altered in its removal.

5.5 Conclusions

Despite the positive qualitative observations, overall the treatments with quercetin and OTC do not appear to have a significant effect on inflammatory cell numbers following laminectomy based upon the parameters examined. The size of the scar tissue left behind follows the trend of being smaller in the experimental treatment groups versus the saline controls; however the measurements of area and widths did not produce statistically significant results when saline controls were compared to experimental treatment groups within the individual time frames studied.

As quercetin and OTC have been shown to reduce inflammation and improve wound healing in other studies (Phan, Sun et al. 2003; Lee, Kim et al. 2006), it is
possible that these treatments have their greatest effect on the cutaneous tissues. Additional studies using cutaneous wound models may result in significantly positive results with these experimental treatments since improvements were observed in the skin aspects of the scars in this study.

Other avenues worth exploration include the possible alterations to the method and dose of drug administration, as well as studying rats of a specific genetic breeding line. It is possible that these changes to the experimental plan may alter the outcome of this study. The morphological differences observed between the experimental treatment groups and the saline controls indicate that further studies are required to determine the efficacy of quercetin and OTC in scar tissue amelioration.
References


