

**The Flavonoid Quercetin and its Potential as Neuroprotectant
in the Therapy of Acute Traumatic CNS Injury:
An Experimental Study**

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To my father, Dr. Werner Schültke.

To everyone who believed in my abilities,

and supported me in my intention

to make some of my dreams come true.

Saskatoon, January 2004

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ABSTRACT

Every year, several thousand individuals suffer spinal cord injury (SCI) in North America, while 1.5 million suffer traumatic brain injury in the U.S.A. alone. Primary mechanical trauma to the CNS is followed by a complex pathology, including vascular dysregulation, ischemia, edema and traumatic hemorrhage. Secondary damage is to a large extent caused by oxidative stress and inflammatory processes, resulting in necrosis and apoptosis of neural cells. If secondary tissue injury could be limited by interference with any of the pathomechanisms involved, preservation of structure and function would increase the potential for functional recovery.

Experiments performed in other laboratories have shown that the polyphenolic flavonoid quercetin acts as an anti-oxidant and anti-inflammatory, reduces edema formation and apoptotic cell death. Quercetin is also an excellent iron chelator. This action profile suggested a high therapeutic potential for acute CNS trauma. Therefore, I used models of both spinal cord injury and head trauma in adult male rats to test the hypothesis that administration of quercetin is beneficial for the therapy of acute traumatic CNS injury. While the primary focus of my work was on therapy of acute traumatic spinal cord injury, quercetin was also evaluated in the settings of chronic SCI and acute head trauma.

I found that, in a rat model of mid-thoracic spinal cord compression injury, 1) administration of quercetin, starting 1 hr after injury and continued every 12 hr, improved recovery of motor function in the hind limbs in more than half of the injured animals to a degree that allowed previously paraplegic animals to step or walk. The minimum quercetin dose that was efficacious was 5 $\mu\text{mol/kg}$. The minimum treatment duration for optimal outcome was determined to be 3 days. In control animals, some spontaneous recovery of

motor function did occur, but never to an extent that allowed animals to step or walk. Quercetin administration was associated with more efficient iron clearance from the site of injury, decreased inflammatory response as reflected in decrease of myeloperoxidase activity and decreased apoptosis of neural cells at the site of injury. 2) Quercetin administered in the same injury model as late as 2 weeks after injury, given in a higher dose than that used for treatment in the acute phase, still resulted in significant recovery of motor function in 40% of the injured animals, although at a lower level of performance, when compared to early onset of treatment. 3) Quercetin administered after moderate fluid percussion brain injury resulted in decreased oxidative stress, as reflected in higher tissue glutathione levels at the site of injury. In animals receiving quercetin, the amplitude of compound action potentials was significantly better maintained at 24 hr and 72 hr after injury than in saline-treated control animals.

My experiments have shown that the flavonoid quercetin is neuroprotective in a rat model of brain trauma and in a rat model of spinal cord injury. My data show that administration of quercetin after CNS trauma promotes iron clearance, decreases oxidative stress and inflammation. Quercetin also decreases apoptotic cell death following neurotrauma. These results suggest that quercetin may be a valuable adjunct in the therapy of acute CNS trauma. There is a possibility that administration of quercetin may be beneficial even in certain settings of chronic CNS trauma. These conclusions are based solely on the results from animal experiments. However, the fact that few adverse reactions have been noted to date in either animal experiments or human trials targeting other diseases is encouraging for the progression to human clinical trials for patients with spinal cord injury.

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LIST OF ABBREVIATIONS

ASIA	American Spinal Injury Association
BBB score	B asso B eattie B resnahan score (for recovery of motor function)
CAP	compound action potentials
cNOS	constitutive nitric oxide synthase
CNS	central nervous system
Fe ²⁺	ferrous iron ion
Fe ³⁺	ferric iron ion
FIM	Functional Independence Measure score
FPI	fluid percussion injury
GSH	glutathione
GSSG	oxidized glutathione
H ₂ O ₂	hydrogen peroxide
HOCl	hypochlorous acid
i.v.	intra-venous
MPO	myeloperoxidase
MRI	magnet resonance imaging
NO [·]	nitric oxide free radical
NASCIS	North American Spinal Cord Injury Study
NYU impactor	New York University impactor (spinal cord trauma model)
OH [·]	hydroxyl free radical
O ⁻	superoxide anion free radical
ONOO ⁻	peroxynitrite
SCI	spinal cord injury
SOD	superoxide dismutase
T	thoracic vertebra
TBI	traumatic brain injury

List of publications and presentations based on work presented in this thesis:

Publications in preparation:

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E.Schültke, R. W. Griebel, B. H. J. Juurlink (2001): The potential of the flavonoid quercetin as acute phase treatment in spinal cord injury. *XXIIIrd International Symposium on Spinal Cord Trauma, Montreal*

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A flavonoid approach to treatment in acute spinal cord injury in a rat model.

3th Annual Meeting of the Canadian Oxidative Stress Consortium, Saskatoon, May 2002

Therapeutic approaches to addressing spinal cord injury.

RUH Neuroscience Rounds, Saskatoon, November 2003

The flavonoid quercetin and its potential as neuroprotectant in the therapy of acute traumatic CNS injury: An experimental study.

Anatomy and Physiology seminar series, January 2004

HYPOTHESES

- 1) Because of its ability to chelate ferrous iron (Fe^{2+}) and decrease the intensity of inflammatory processes, administration of the flavonoid quercetin will promote significant functional recovery after neurotrauma.
- 2) Administration of quercetin in the acute phase after injury is neuroprotective, while no neuroprotective effect is expected with administration beyond the acute phase of injury.

These hypotheses were tested in models of mid-thoracic spinal cord compression injury and head trauma in the adult rat.

Chapter 1

The Pathology of Spinal Cord Injury

From a clinical aspect, damage and recovery after acute trauma to the CNS include three characteristic phases. The acute phase, immediately following the mechanical impact, the intermediate, the post-acute phase and the chronic phase (Herrmann and Stancil, 1977; Tator, 1995 and 1998; Hulsebosch, 2002). The acute phase is characterized clinically by a state of spinal shock and neuropathologically by an increase in the volume of tissue injury, caused by a complex of pathological processes referred to as secondary injury (Tator and Fehlings, 1991; Tator and Koyanagi, 1997; Carlson et al., 1998; Beattie and Bresnahan, 2000). The term secondary injury is used to distinguish the damage caused by the primary injury, describing the original mechanical impact, from the damage caused by subsequent changes in the extracellular milieu (Tator, 1995; Sekhon and Fehlings, 2001). Since the intensity of characteristic pathologic processes involved in injury varies considerably over the course of time, it has become customary to distinguish between acute and chronic spinal cord injury.

1.1 The epidemiology of spinal cord injury

The reported incidence of spinal cord injury varies between 15 and 71 cases per million, depending on the mode of reporting (Tator, 1995; Sekhon and Fehlings, 2001). Studies reporting higher numbers might describe the situation more accurately, because they take into account immediate deaths before hospital admission (Sekhon and Fehlings, 2001; Kirshblum et al., 2002). It is estimated that of the 12,000 new cases of paraplegia and quadriplegia that occur in the United States each year, 4,000 die before reaching the hospital (Sekhon and Fehlings, 2001). According to the estimates of the Canadian Paraplegic

Association, about 35 new cases per million population are seen each year in Canada. Men are afflicted four times as often as women, and about 60% are 30 years or younger (Go et al., 1995). The consequence of those statistical data, besides putting figures on personal tragedies, is that for the afflicted patients the major part of economically active life is severely compromised. A long-term outcome study of patients aged 25-34, who had suffered acute traumatic spinal cord injury (SCI) while still in the pediatric age group showed that the employment rate was only 54%, while the employment rate in the general population for the same age group was 84% (Vogel et al., 1998). For society as a whole, this results in double economic loss: not only are the economic contributions of those young people lost, but heavy expenses are required to pay for special life long care. In 1990, the costs incurred for society from acute and long-term care of surviving spinal cord injury victims were estimated at 4 billion dollars annually in the U.S.A. alone (Stripling, 1990). Before the practice of intermittent catheterization and skin care through positional changes were introduced by Ludwig Guttman and others in the 1940s, post-hospitalization mortality of patients with spinal cord injury was mainly due to sepsis and urologic complications (Thomson-Walker, 1937; Sutton, 1973). Mortality from those causes has decreased significantly since. While respiratory complications are now perceived as the leading cause of death in patients admitted with SCI, the runners up are heart disease, septicemia, pulmonary emboli, suicide and unintentional injuries (DeVivo et al., 1989). With regard to specialized care of the patient with spinal cord injury, prevention of medical complications is the primary focus, starting on the day of injury. Measures include pressure relief for the skin to prevent pressure sores as potential source of infection, thromboembolism prophylaxis, prevention of gastric ulcers,

Foley catheter drainage to prevent urinary retention as source of infection and urosepsis, and bowel care to prevent colonic impaction (Kirshblum et al, 2002).

While survival of patients after reaching the hospital alive has increased dramatically since the 1940's, chances of reaching the hospital alive with spinal cord injury have only slightly improved. As an example, a study of incidence of spinal cord trauma in Olmstead County, Minnesota, U.S.A., showed the overall incidence of acute traumatic SCI tripling between 1935 and 1974. Yet, the number of patients reaching the hospital alive has only increased three-and-one-half times over the same period in the same jurisdiction (Griffin, 1985; Sekhon and Fehlings 2001). Having said this, it is worth mentioning that the percentage of complete spinal cord injury on arrival at the hospital has been significantly decreasing over the last decades (Sekhon and Fehlings, 2001; Tator et al., 1993). Increased public awareness of risk factors leading to head trauma and spinal cord injury, the introduction of mandatory use of safety belts and the installation of air bags in cars as well as widespread First Medical Aid instruction have certainly contributed to the decreased incidence of complete spinal cord injury. While Kossuth is credited with having developed the concepts of protecting and immobilizing the cervical spine during extrication from a vehicle and rescue from the accident scene, many others have championed the idea (Kossuth, 1965 and 1967). Although, for ethical reasons, no clinical trial yielding class I or class II evidence to support cervical spine immobilization is conceivable, the fact that the percentage of patients with multiple injuries who suffered complete spinal cord injury decreased from 55% in the 1970s to 39% in the 1980s speaks for itself (Garfin et al., 1989). Since the time period of declining incidence of complete spinal cord traumata coincides with the popularization of cervical spine immobilization during the pre-hospitalization phase, this

could be considered indirect evidence for the ability of immobilization to prevent delayed mechanical injury to the spinal cord. The most important predictive factors of survival after acute traumatic SCI are patient age, the level of injury and neurologic grade (Claxton et al., 1998). With grading systems, such as the American Spinal Injury Association (ASIA) motor index and FIM (Functional Independence Measure) score, standardized methods of assessment have been developed for assessment of the level of temporary neurological dysfunction as well as for neurological recovery.

The most common mechanism of spinal cord injury is fracture-dislocation, with anterior fracture-dislocation being more common than either posterior or lateral injuries. Although the overall percentage of cervical spine injuries is higher than that for thoracic injuries, the percentage of associated complete spinal cord injury is, at 77.5% (vs. 60.4% for cervical and 64.7% for lumbar spine), highest with injury to the thoracic spine (Tator, 1983).

1.2 The value of evidence-based medicine, clinical trials and guidelines

With increasing specialization in the medical field and a host of new data available in ever shorter periods, there is a necessity to create treatment guidelines which cover the most common clinical situations. Besides being informative, such guidelines can also be seen as important protection against the unfortunately increasing tendency of patients to use litigation in order to achieve compensation for real or perceived shortcomings in medical treatment. Guidelines should be based on clinical evidence supporting the claim that the therapeutic approaches recommended are, according to current knowledge and opinion, the best available to the patient. Medical evidence comes in different categories, ranging from anecdotal reports to large-scale randomized trials. While anecdotal reports may pique our interest and, occasionally, trigger a research project, it is evidence from the large randomized trials that is increasingly used to develop guidelines for clinical practice. In 1990, some key concepts for the creation of guidelines for clinical practice were published on behalf of the Institute of Medicine in Washington, DC (Field and Lohr, 1990). Those key concepts included the request 1) that a thorough review of the available scientific literature should precede the development of guidelines, 2) that claims made in scientific publications should be evaluated with regards to validity, and 3) that the strength of recommendations in the guidelines should reflect the strength of this evidence. This sounds like common sense, but especially in medico-legal disputes it can be essential whether any specific treatment approach is considered standard, recommended or optional by the medical community and its advisory boards. Also, it was requested that empirical evidence take precedence over expert judgement. Agreements are required to judge the value of evidence presented in

clinical trials. Currently, in the concept of evidence-based medicine, evidence presented in any study is classified in three levels. Strongest evidence is signified by level I, which describes evidence arising from randomized, placebo-controlled clinical trials. In this type of study, prospectively, adherence to strict study protocols and availability of a contemporaneous control group all reduce the sources of systematic bias (Hadley et al, 2002). Random error is reduced by the randomization process, since this reduces the chance that unrecognized aspects of triage might influence the outcome. Good but somewhat weaker evidence is represented at level II, which describes the value of prospective non-randomized cohort studies and case-control studies. In prospective cohort studies, patients with similar injury patterns are placed in either treatment group consciously, and the difference in outcome is studied. While case-control studies are typically retrospective, patients are grouped by outcome, and research focuses on a possible link between the different outcomes and different treatments. Class III evidence is presented by published case series, case reports and expert opinions based on observations and personal experience.

Another important consideration is assessment of the strength of diagnostic tests used in a study. To yield valuable results, a test must be reliable (come up with similar results under similar circumstances), valid (reflecting the true state of affairs or comparable results when measured against a “gold standard”) and accurate (acceptable ability to distinguish between patients who have and those who do not have the disorder which is tested for) (Hadley et al, 2002). Sensitivity, specificity, positive and negative predictive values therefore are important characteristics of a diagnostic test.

Furthermore, an important criterion to classify the evidence of any study is the strength of patient assessment for the study, both for the initial condition and outcome.

Again, we are looking at reliability, which is assured by calibration of instruments in regular intervals or verification of agreement between different observers of the same process. To assure that inter-observer agreement did not occur purely by chance, a concordance index (kappa index) has been developed (Cohen, 1960; Landis and Koch, 1977). The Bayesian 2x2 table can be used in the following way to calculate the concordance index (Table 1.1):

Observer 1	Observer 2		
Yes	Agree (a)	Disagree (b)	(a+b) = f ₁
No	Disagree (c)	Agree (d)	(c+d) = f ₂
	(a+c) = n ₁	(b+d) = n ₂	(a+b+c+d) = N

Table 1.1 Bayesian table to calculate concordance index.

From: Hadley et al., 2002

From these numbers, kappa is calculated by:

$$\text{Kappa} = \frac{N(a+d) - (n_1 f_1 + n_2 f_2)}{N^2 - (n_1 f_1 + n_2 f_2)}$$

or

$$\text{Kappa} = \frac{2(ad - bc)}{(n_1f_1 + n_2f_2)}$$

The following values for kappa refer to qualitative assessment of inter-observer agreement:

Kappa value	Level of evidence	Strength of agreement
< 0	Class III evidence	Poor
0 – 0.20	Class III evidence	Slight
0.21 – 0.40	Class III evidence	Fair
0.41 – 0.60	Class III evidence	Moderate
0.61 – 0.80	Class II evidence	Substantial
0.81 – 1.00	Class I evidence	Almost perfect

Table 1.2 Qualitative assessment of inter-observer agreement.

From: Hadley et al., 2002

1.3 The acute phase of traumatic spinal cord injury

In the acutely traumatized spinal cord, several mechanisms of destruction are observed, contributing to primary or secondary loss of function and structure. The primary injury, caused by the initial traumatic impact on the spinal cord, includes mechanisms such as contusion, compression and partial or complete transection. Instability of membrane function and frank membrane disruption, vascular damage with consecutive hemorrhages and ischemia follow. Primary complete transection of the spinal cord appears to be relatively infrequent, compared to presentations containing a blunt injury with elements of contusion, compression and possibly partial transection (Kakulas, 1984). However, secondary pathomechanisms may result, at a later point, in complete loss of functional spinal cord tissue across the complete cross-sectional surface of the spinal cord at the site of injury (Tator and Rowed, 1979; Fehlings and Tator, 1988). Those pathological processes have been summarized under the term 'secondary injury'. Hematomas obviously contribute to internal or external spinal cord compression at macroscopic level, thus increasing the vicious circle of ischemia, acidosis and edema (Hughes, 1988). However, a number of pathologic processes at the cellular level, such as inflammation, lipid peroxidation as expression of oxidative stress, calcium influx and excitotoxic cell death contribute further to the developing tissue injury (Sharma and Winkler, 2002; Hausmann, 2003; Wingrave et al., 2003). While ischemic and excitotoxic cell damage may be difficult to quantify in the clinical situation, the consequences of those processes are apparent, with some delay, as edema in imaging procedures and as secondary loss of function detected during neurological assessment.

Initial management of acute traumatic spinal cord injury includes establishment of oxygenation, circulation, radiographic evaluation for spinal instability and re-establishment of spinal alignment (Kirshblum et al., 2002). The non-operative management of patients with spinal cord injury should be directed at the prevention of secondary injury and prevention of systemic physiological derangement resulting from the injury to spinal cord and adjacent structures of the nervous system, such as the sympathetic chain. Immobilization of the spine, to prevent further mechanical trauma, adequate oxygenation and sufficient tissue perfusion are presently the mainstay of treatment. The problem with satisfying the latter requirement is that, while there is sufficient knowledge regarding the blood pressure necessary for adequate perfusion of kidneys, brain and viscera, hardly any data exist to indicate the optimal blood pressure for adequate perfusion of the spinal cord (Ball and Nockels, 2001). Therefore, inadequate support of spinal cord perfusion might contribute significantly to secondary injury after SCI.

Frequently encountered clinical problems in patients with spinal cord injury include respiratory insufficiency, apnea, paralysis and spasticity. Contributing is the following spinal cord pathology:

1) motor control: Transmission of stimuli and voluntary control of muscle function below the level of injury are lost because of injury to the anterior and lateral corticospinal tracts as well as extrapyramidal pathways.

2) sensation: Proprioceptive impulses from muscle spindles and receptors in joints do not reach the brain due to injury to the dorsal columns.

3) local reflex circuits: functional loss of local reflex circuits is due to destruction of gray matter at the site of injury.

Autonomic nervous system dysregulation: A good summary of the general effects of acute traumatic spinal cord injury on the autonomous nervous system can be found by Cull and Hardy (1977). In the healthy, uninjured spinal cord, spinal cord function is controlled on two levels: autonomous spinal cord function, based on local circuitries, and suprasegmental control. If the spinal cord is transected above T1, suprasegmental control of the sympathetic nervous system is lost, while transection below T2 leaves some of the sympathetic system intact, though impaired. Immediately after spinal cord injury, spinal cord reflex activity below the lesion transecting the cord is lost transiently. This state, also termed ‘spinal shock’, is transient and may continue as long as two or three months after injury. One of the consequences is the loss of vasomotor tone, with dependent pooling of blood. Cardiovascular output is reduced, which causes hypotensive crises in the newly injured. At the same time, autonomic hyperflexia is seen in those patients with cord injuries at T5 level and above. Characteristic is a marked rise in blood pressure as consequence of a massive sympathetic reflex response to stimuli arising in the pelvis from overdistension of bladder and rectum. While the loss of thoracic nerves from T1-6 results in paralysis of the intercostal muscles, the functional loss of T7-L1 nerves is seen in the abdominal muscles (Cull and Hardy, 1977).

Excessive sweating, reflecting post-traumatic changes in thermoregulation, is encountered in both human patients and experimental animals in the first few days after trauma. “Sweating is the result of stimulation of the sympathetic nervous system, and may occur on a segmental reflex basis as a result of over distension of bowel and bladder” (Cull and Hardy, 1977).

Problems associated with micturition: In the adult healthy, uninjured subject, the bladder is under cortical control. This inhibits or facilitates the spinal reflex activity in the conus medullaris (S3-4) and thus allows continence. Because of the loss of cortical influence and the initial flaccidity of the detrusor muscle immediately after trauma, artificial drainage of urine by catheterization is required. However, once the areflexic period of spinal shock has passed, the bladder reverts to a primitive pattern of emptying, and reinstatement of voiding may occur. Bors classified bladder function after spinal cord injury into three categories.

1. *Upper motor neuron type:* This is typical for lesions above the conus medullaris (S2-3). Lesions result in a reflexic, automatic, spastic voiding pattern. Credé maneuver or straining is usually not required, and bladder capacity is limited to below 400 cc (normal capacity 500 – 600 cc).

2. *Lower motor neuron type:* This type of bladder dysfunction is seen with lesions to the conus medullaris, or to the lower sacral roots. The spinal reflex arc needed for detrusor tonus and integrated detrusor emptying contraction is interrupted. Bladders are flaccid, have an increased capacity, up to 1000 cc, and depend on external pressure for emptying.

3. *Mixed type:* With this type of lesion, bladders have the partial ability to empty by reflex, and may require assistance by Credé maneuver. This is possible due to the partial innervation of the bladder by pelvic or pudendal nerves. True reflex automaticity is lacking. The musculature can not be categorized as either spastic or flaccid. (Cull and Hardy, 1977).

1.3.1 The secondary injury theory

Secondary injury has been recognized as a central theme in the natural course of traumatic CNS lesions (Tator, 2002). One of the key observations concerns the fact that a significant number of cells, both in the epicenter of injury and in adjacent tissue sections, apparently survive the initial impact, but die at a later stage due to deterioration of the extracellular milieu and loss of membrane integrity. The concept of secondary injury is based on the observation that, hours to weeks after the trauma, cell death by necrosis or apoptosis is induced in cellular structures previously undamaged by the primary mechanical impact, and that damage becomes permanent in tissue structures which potentially could have recovered (Crowe et al., 1997; Shuman et al., 1997). The fact that the volume of tissue injury is increasing continuously for a certain period of time after primary trauma to the spinal cord has been reported as early as 1911 by Allen and 1923 by McVeigh. Allen found that, in a dog model of acute traumatic spinal cord injury, removal of the post-traumatic hematomyelia resulted in significantly improved recovery of motor function (Allen, 1911). Since then, increasingly sophisticated investigation techniques have allowed more and more detailed dissection of the pathologic cellular mechanisms that result in secondary tissue injury. Numerous experiments have resulted in more detailed knowledge about the secondary tissue injury developing at the site of injury, in immediately adjacent and in more distant tissue sections. Mechanisms contributing to increase the secondary damage include contusional hemorrhage, vascular dysregulation and ischemia, edema formation, oxidative stress and inflammatory processes. Damage caused by those pathomechanisms will subsequently contribute to a significant secondary increase of lesion volume in both acute spinal cord

injury and head trauma (Tator and Fehlings, 1991; Wahl et al., 1993; Carlson et al., 1998; Juurlink and Paterson, 1998; Schnell et al., 1999; Tator, 2002).

It has been shown in rat as well as in monkey models that both neurons and glia can die by apoptosis (Beatty et al., 1998; Crowe et al., 1997; Liu et al., 1997). Apoptotic cell death is expected to contribute to the increasing volume of secondary injury, probably weeks to even months after injury (Johnson et al., 1995). Activation and consequent apoptosis of microglia have been shown in a rat model (Shuman et al., 1997). Whether microglial activation is a cause for or rather a consequence of apoptosis of oligodendroglia, is still debated. The majority of apoptotic cells have been found in the white matter tracts, and more so in ascending than in descending tracts. This has been shown in the animal model as well as in human spinal cord injury (Hayes and Kakulas, 1997; Shuman et al., 1997; Emery et al., 1998; Beattie et al., 2000). Those apoptotic cells are typically found in oligodendrocytes and microglia, in regions of Wallerian axonal regeneration. This loss of oligodendrocytes probably contributes to the demyelination seen after acute spinal cord trauma.

A consequence of mechanical trauma and ischemia on spinal cord tissue consists in the release of glutamate into the extracellular space from injured neurons and astrocytes (Strjibos et al., 1996; Benveniste et al., 1984). The increase of extracellular glutamate levels leads to a significant increase of intracellular Ca^{2+} levels, which can stimulate the expression of nuclear cell death genes (Kroemer et al., 1995). Increased intracellular Ca^{2+} levels have been associated with ROS (reactive oxygen species) production and phospholipid membrane degeneration (Werling and Fiskum, 1996), and with increased permeability of the inner mitochondrial membrane (Bernardi et al., 1998). The latter process has been associated with

the production of reactive oxygen species and osmotic mitochondrial swelling, promoting the release of apoptogenic mitochondrial proteins (Fiskum, 2001).

The next consideration is given to the processes of ischemia and re-perfusion injury. Ischemia and re-perfusion phenomena initially were intensely studied in the context of stroke and cardiac disease (Fiskum, 1985; Zweier, 1988; Das et al., 1989). It was determined that mitochondria are the main location of free radical production during the reperfusion phase after ischemia, consequent to a reduction of components of the electron-transport chain and a decrease in superoxide dismutase activity (Grill et al., 1992; Szweda et al., 2001; Ferrari, 1996). In the normal state, the electron transport chain in the mitochondria controls the redox energy required to generate the mitochondrial membrane potential. Electrons derived from various substrates are stored in the substrate NADH, from where about 95% of the electrons are passed down the respiratory chain to reduce oxygen to water. However, even in the undisturbed state, some electrons combine with oxygen at intermediate steps of the respiratory chain to produce the superoxide radical $O_2^{\cdot-}$ (Kowaltowski and Vercesi, 2001). Under physiological circumstances, the superoxide anion is converted by superoxide dismutase to hydrogen peroxide, an apparently cytoprotective process (Fridovich, 1978; Bielski, 1985). The hydrogen peroxide then is reduced to water and molecular oxygen by catalase and glutathione peroxidase, the latter of which uses glutathione as an electron donor (Emerit, 1988; Sies and Moss, 1978). In the case of ischemia, the decrease of cytoprotective dismutase activity would lead to an increase in superoxide anions. Increased intracellular superoxide anion levels will lead to leakage of the radical into the extracellular space, where superoxide dismutase levels are low (Emerit, 1988). While nitric oxide (NO) in itself is not toxic, it will form peroxynitrite (ONOO⁻) when it interacts with superoxide anions, a process

which in turn promotes mitochondrial lipid membrane peroxidation (Gadelha et al., 1997). Nitric oxide is a smooth muscle relaxing factor responsible for vasodilator response, while superoxide anions are considered potent vasoconstrictors, mainly because they scavenge NO (Marleatta, 1989; Moncada et al., 1989). Unchecked increase of superoxide anions, therefore, might cause considerable vasoconstriction, thereby aggravating the ischemic state (Gryglewski et al., 1986; Ischihara et al., 2001). The production of superoxide anions and other free radicals during the reperfusion phase is paralleled by an increased rate of lipid peroxidation (Kramer et al., 1994; Ambrosio et al., 1993; Cordis et al., 1993). Many of the compounds produced by the breakdown of polyunsaturated fatty acids in the process of lipid membrane peroxidation, such as aldehydes, alkenals and hydroxyalkenals, have been shown to be cytotoxic both in vitro and in vivo (Esterbauer et al., 1991). Therefore, it can be assumed that ischemic conditions during the primary impact, as well as consequent to developing edema, prime mitochondria for the production of free radicals, which ultimately results in lipid membrane peroxidation. Lipid membrane peroxidation results in structural and functional damage to the mitochondria and other cellular structures, so that the vicious circle is perpetuated and the volume of the tissue damage increases (Gogvadze et al., 2003). Oxidant species released by neutrophils attracted to the site of injury further contribute to secondary tissue injury.

The first days after CNS injury are characterized by an influx of neutrophils and macrophages into the tissue at the site of injury and adjacent spinal cord segments in both animal model and human patients (Matteo and Smith, 1988; Anderson, 1992). Inflammation, lipid membrane peroxidation as expression of oxidative stress and excitotoxic processes are key mechanisms of secondary CNS injury, with inflammation being perhaps the most

controversial problem. Inflammatory responses include the invasion of traumatized parenchyma by neutrophils from the blood stream, commencing only hours after the injury with a peak at 24 hr, and the presence of phagocytic cells at the site of injury. Regarding the latter, we need to distinguish between blood-born macrophages and phagocytic cells derived from activated microglia. Microglia is activated as early as 12 hr after SCI, while monocyte infiltration is delayed over a period of days, with a peak around five days after injury (McTigue, 2000). It is not possible to distinguish histologically between cells with glial origin and those generated from circulating monocytes once they have reached the phagocytic stage. Popovich and colleagues (1999) developed a technique by which they were able to deplete circulating monocytes after spinal cord contusion injury in a rat model, without affecting resident microglia. This resulted in significant tissue sparing and improved recovery of locomotor function.

Neutrophils, when stimulated, generate potent reactive oxygen species (Hampton et al., 1998; Winterbourn et al., 1985; Badwey and Karnovsky, 1980; Badwey et al., 1991). Superoxide anion radicals ($O_2^{\cdot -}$) are generated by activation of the enzyme NADPH oxidase and subsequently converted to other reactive species, including H_2O_2 (Roos, 1991a and 1991b). The oxidant activity of H_2O_2 is significantly enhanced by the action of myeloperoxidase, a hemoprotein enzyme usually stored in the granules of neutrophils. Myeloperoxidase has been described as key regulator in the oxidant production by cellular mediators of inflammation (Kettle and Winterbourn, 1997). In the presence of Cl^- or other substrates acting as electron donors, myeloperoxidase catalyzes the reaction of hydrogen peroxide and chloride anions, generating chloramines and hypochlorous acid (HOCl), which has a reactivity about two orders of magnitude higher than that of H_2O_2 alone (Weiss et al.,

1982; Weiss, 1985; Thomas et al, 1983; Pincemail et al., 1988; Rodrigues et al., 2002; Gaut et al., 2001). This reaction appears to be dependent on the availability of ferric iron (Fe^{+3}) (Rao et al., 1988). Hypochlorous acid, when released into the extracellular space, will react with tissue structures adjacent to the site of injury and thus increase the volume of secondary tissue damage (Prutz, 1996; Selloum et al., 2001) (Fig. 1.2). Chlorinated structures can be restored to their unchlorinated state by reduction with GSH (Prutz, 1998). However, the increased demand for repair will quickly deplete the cytoprotective GSH pool after CNS injury, so that the potentially reversible damage becomes permanent (Juurlink and Paterson, 1998).

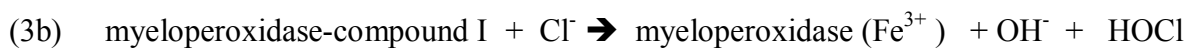
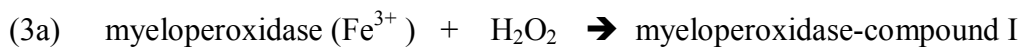


FIGURE 1.1: Neutrophils, when activated, generate reactive oxygen species and hypochlorous acid. Hypochlorous acid causes tissue damage.

Busse and colleagues have demonstrated that flavonoids inhibit the release of reactive oxygen species by human neutrophils (Busse et al., 1984).

While measurement of protein carbonyl content offers an indirect method to assess the extent of cellular protein reaction with HOCl (Chapman et al., 2000), most commonly the capacity of myeloperoxidase to form hypochlorous acid from hydrogen peroxide and chloride ions is used. Myeloperoxidase activity has become an accepted marker of neutrophil activity. Carlson and colleagues have assayed myeloperoxidase activity in the spinal cord of rats between 4 and 48 hr after acute spinal cord contusion injury (Carlson et al., 1998). They found that MPO activity peaked at 24 hr after injury, with the center of activity found between 4 mm rostral and caudal to the site of injury. Quercetin has been shown in vitro to decrease myeloperoxidase activity in human neutrophils in a dose-dependent manner, thereby intercepting and limiting the development of pathology just described (Pincemail et al., 1988).

In order to understand the complexity of post-traumatic inflammatory processes, we should distinguish between two phases of inflammatory response, an 'early' and a 'late' phase (Bethea, 2000). The 'early' phase begins within hours of the injury, includes elevation of TNF- α and monocyte chemoattractant protein (MCP-1) and peaks approximately 1 day later (coinciding with the peak of neutrophil presence at the injury site). The 'late' inflammatory response is characterized by the infiltration of macrophages into the site of injury, which peaks between days two and three post injury (Bethea, 2000). The presence of macrophages in the spinal cord can persist for several weeks, even months after the injury. Macrophages secrete growth factors, proteolytic enzymes and cytokines essential for the remodeling process after SCI. T-lymphocytes are essential for activation of macrophages,

and in fact it has been shown in a rat model that they enter the injured tissue as early as 2-3 days after injury, with a peak presence at 1 week after injury (McTigue, 2000). The presence of T-cells in the spinal cord tissue persists for several weeks after the injury (Popovich et al., 1997). It has been shown that the influx of neutrophils and T-cells and the presence of phagocytic cells corresponds temporally with the expression of chemokines, a family of pro-inflammatory cytokines. In the same animal model (McTigue et al. 1998), it was suggested that those cells may be responding to chemoattractive molecules produced in the injured spinal cord tissue (Bethea, 2000; McTigue, 2000). Post-traumatic inflammatory processes are characterized by influx of neutrophils and macrophages, and by the activation of residential phagocytic cells. Proteases and oxidant substances are released into the surrounding tissue (Bank et al., 1999). Oxidative stress leads to lipid membrane peroxidation, resulting in loss of polyunsaturated fatty acids from membrane phospholipids. The loss of polar species from the plasma membranes contributes to increased membrane rigidity. Both the changes in membrane permeability and fluidity lead to decreased integrity of the plasma membranes (Farooqui and Horrocks, 1998). From this point, cells may either recover or die.

A multitude of cytokines is involved in the initiation, perpetuation, and regulation of inflammatory processes. Cytokines are found in the spinal cord parenchyma after injury as well as in CSF and blood. They may be associated with either pro-inflammatory action like tumor necrosis factor-alpha (TNF- α), interleukin-1 (IL-1) and interleukin-6 (IL-6) or anti-inflammatory action, such as interleukin-4 (IL-4), interleukin-10 (IL-10) or interleukin-13 (IL-13) (Bethea, 2000). TNF- α , as an example of a pro-inflammatory cytokine, was found to be elevated as early as 1 hr after injury and persisted for up to 7 days in a study by Bethea

and colleagues (2000). Pan and his colleagues (1999) demonstrated an increase of TNF- α at the injury site within minutes after injury, peaking and plateauing between 1 and 5 days. Potential sources for TNF- α are neutrophils, macrophages and activated microglia (Bethea, 2000). While the role of TNF- α in the inflammatory processes associated with CNS injury has been controversial with experimental results derived from tissue cultures, in vivo studies demonstrate that it is a potent mediator of microgliosis, astrogliosis and cell death. A study by Lavine and colleagues (1998) demonstrated that neurological outcome was improved after ischemia-reperfusion injury in a rat model with administration of a neutralizing antibody to TNF- α . Many pathomechanisms observed where inflammatory processes are active can be related to oxidative stress in damaged tissue structures.

It has been shown that in many cases of spinal cord injury the volume of secondary damage by far exceeds that caused by the primary injury. Therefore, we believe that the prevention of secondary injury is the most important concept in the treatment of patients with spinal cord injury. The most hotly debated therapeutic agent in this regard is the drug methylprednisolone.

When methylprednisolone was introduced in the late 1980's as standard pharmacologic treatment for the early phase after spinal cord injury, high hopes by both patients and physicians were set on this drug. Studies in cats had shown tissue preservation and significantly improved recovery with administration of methylprednisolone after acute traumatic spinal cord injury (Means et al., 1981). The neuroprotective effect of the compound was ascribed to attenuation of lipid peroxide formation and, as a consequence, increased Na⁺-K⁺-ATPase activity, hyperpolarization of motor neuron resting membrane potentials and accelerated impulse conduction along the myelinated portion of the motor axon (Braugher

and Hall, 1982). Ten years later, enthusiasm has cooled considerably. Voices from both basic scientists and clinicians have raised concerns that adverse effects of high-dose administration of the corticosteroid might considerably outweigh the potential benefits to patients (Nesathurai, 1998; Qian et al., 2000; del Rosario Molano et al., 2002). Three major clinical multicenter studies have been performed over the last decade, to investigate the clinical effects of high-dose methylprednisolone administration after acute traumatic spinal cord injury. NASCIS I focused on recovery of motor function, light touch and sensation after administration of methylprednisolone. No significant benefit from the drug was detected, but this was believed to be due the fact that the dose delivered had simply been too small to be effective. This problem was meant to be amended with NASCIS II, which was conducted as randomized, placebo-controlled study and included 487 patients. Treatment with methylprednisolone for 24 hr, started within 12 hr after injury, was compared to treatment with naloxone or placebo. Again, no significant benefit was seen with treatment, but from a post-hoc analysis it appeared that a small gain in the motor and sensory scores could be attributed to treatment with methylprednisolone. Other studies, trying to replicate those results and verify the claims, came up with controversial results. While, in one study, improved muscle function was seen with methylprednisolone treatment when tested at 6 months after injury (Otani et al., 1996), no improvement was found in another study (Petitjean et al., 1998). NASCIS III, with a trial protocol almost identical to NASCIS II (although lacking a placebo group), included 499 patients and tested the hypothesis that outcome might be improved with an extended treatment period. This hypothesis could not be upheld. A modest, statistically not significant improvement on the Functional Independence Measurement scale (FIM) at 48 hr after injury ($p = 0.08$) was outweighed by increased

prevalence of both sepsis and pneumonia after 48 hr treatment. While some authors feel that NASCIS II and III present Class I and Class II evidence to give general support for administration of methylprednisolone to patients after acute traumatic SCI (Bracken et al., 1997; Bracken and Holford, 2002), others feel that both studies present at best weak evidence for the therapeutic efficacy of methylprednisolone after acute traumatic SCI (Benzel, 2002; Rabchevsky, 2002; Coleman, 2000; Hurlbert, 2000; Short et al., 2000, Nesathurai, 1998). Some authors have warned that, if given outside the very limited therapeutic window of 3 – 8 hr after injury, the corticosteroid has outright deleterious effects (del Rosario Molano et al, 2002; Fehlings, 2001; Hurlbert, 2001). Advocates of methylprednisolone therapy try to justify their recommendations by pointing to the fact that, while there is no improvement of functionality as measured by FIM, neurological improvements might have been registered if the more sensitive ASIA (American Spinal Injury Association) scale would have been used. They hold that any improvement, even if not of any functional consequence, satisfies the requirement of cost-effectiveness (Bracken and Holford, 2002). The results of recent animal experiments causes us to consider whether methylprednisolone is becoming another case in which the translation of research from bench to bedside failed because circumstances critical for successful action of the compound were overlooked. Haghghi and colleagues (2000) published their results of an animal study (rat) in which methylprednisolone, administered after 45 g compression injury of 2 minutes duration at the mid-thoracic spinal cord, had no impact on neurological function. On a microscopic level, lesion and cavity volumes were even significantly larger in the spinal cords of animals treated with methylprednisolone, as compared to saline-treated controls. Published by the team of Dr. Wise Young, who initially promoted the widespread use of methylprednisolone in the therapy of spinal cord injury, was

a study clearly stating that in an animal model (adult rat) the therapeutic window of methylprednisolone for reduction of lesion volume is less than 30 minutes (Yoon, 1999). We might expect that pathology develops considerably quicker in a small animal like a rat, compared to primates. But, since it is difficult to make a valid estimate with regard to the correlation of time spans between rats and human patients, a very short therapeutic window of a drug can be expected to cause problems in human clinical trials. Within the last five years, several reviews and meta-analyses of studies using methylprednisolone in the setting of traumatic spinal cord injury have been published worldwide. The tenor of those publications is clearly a warning that, while very little benefit for the patient can be expected, the risk of serious adverse effects is not negligible, and therefore this treatment option is not recommended (Pointillard et al., 2000; Short et al., 2000; Matsumoto et al., 2001; Short, 2001; Citerio et al. 2002). Considering the contradictory interpretations of the results from the major clinical trials involving methylprednisolone, an expert panel has agreed that, “given the devastating impact of spinal cord injury and the modest efficacy of methylprednisolone, clinical trials of other therapeutic interventions are urgently required” (Fehlings, 2001). However, based on current analysis, the recommendation given by the Committee of the Canadian Spine Society and the Canadian Neurosurgical Society was adopted, which declared that the 24 hr treatment with methylprednisolone, started within 8 hr after spinal cord trauma is no longer considered standard treatment, but rather a treatment option left to the discretion of the treating physician (Hugenholtz et al., 2002; Hugenholtz, 2003). Although the high hopes set for the drug were not fulfilled, the clinical trials and the ensuing debate helped to abandon the century-old dogma that spinal cord injury was permanent and not accessible to therapy.

1.3.2 The concept of oxidative stress

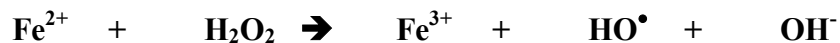
In his book '*La Pression Barometrique*', published in 1878, Paul Bert gave what is believed to be the earliest comprehensive overview about the subject of oxygen toxicity in biological systems (Haugaard, 1968). Bert pointed out that the nervous system is especially vulnerable in this respect. Vital to the understanding of Bert's observations was the concept of oxygen activation. In 1929, Mulliken published results from one of his spectral analysis experiments, concluding that oxygen could assume an excited state through a change in its electronic configuration. Mulliken's conclusion was later confirmed by the experiments of Herzberg (1934 and 1950) and Mallet (1972). As explained by Michaelis (1949), activation of oxygen leads to formation of singlet oxygen ($^1\text{O}_2$) and a number of free radicals. Free radical formation involves a reductive process, by which the oxygen gains electrons. Reduced oxygen reacts with other molecules. Reactive oxygen-based molecules have been termed 'reactive oxygen species' and include superoxide anions ($\text{O}_2^{\bullet-}$) and hydroxyl radicals (HO^{\bullet}), hydrogen peroxide (H_2O_2), peroxy radicals (RO_2^{\bullet}) and organic hydroperoxides (ROOH). The production of reactive oxygen species can be caused by a variety of processes, including trauma, infection, electrochemical or photo effects. In the setting of CNS tissue injury, the most likely routes of oxygen activation are enzymatic processes and singlet oxygen production from hydrogen peroxide. Singlet oxygen and reactive oxygen species have been found to influence a number of biological processes, including enzymatic reactions in the mitochondrial respiratory chain (Chance et al., 1979; Nohl and Hegner, 1978) and phagocytosis (Klebanoff, 1980; Badwey et al., 1980; Allen et al. 1974).

Particularly important during lipid peroxidation is the formation of hydroperoxides from peroxy radicals. The type of reaction is similar to that of the Fenton reaction, but

organic hydroperoxides are less reactive than hydrogen peroxide. Hydroperoxides can be scavenged by the anti-oxidant glutathione peroxidase, but not with catalase. Their presence can be detected spectroscopically at 234 nm. The presence of ferrous and ferric iron is a key component of these reactions:



It is assumed that the Fenton reaction, driven by hydrogen peroxide and ferrous iron ions, is a key process in the bactericidal action of neutrophils and consequent phagocytosis (Babior, 1982). Hydroxyl radicals in injured tissue are likely to arise by way of the Fenton reaction (metal ion with hydrogen peroxide) (Halliwell and Gutteridge, 1985; Walling, 1975):



The hydroxyl (OH[•]) radical is one of the most reactive free radicals known. It is more reactive than either singlet oxygen (Wilkinson and Brummer, 1981), superoxide anion (Bielski et al., 1985) or peroxy radicals (Howard, 1984). Hydroxyl ions in biological systems react by addition to unsaturated sites or by abstraction of hydrogen. Most of this knowledge is derived from radiation biology studies (Singh and Singh, 1982).

The phenomenon of lipid peroxidation had been first observed in the food industry, with descriptions from as early as the 19th century, when industrial research led to the discovery that tocopherol was a potent anti-oxidant for fat-containing food (Miquel, 1989).

Tocopherol was later found to also act as anti-oxidant in the lipid containing membranes of mammalian cells (Evans and Bishop, 1922), an effect which was complemented by water-soluble anti-oxidants such as vitamin C (Szent-Gyorgy, 1928), uric acid (Ames et al. 1981) and glutathione (Viña et al., 1989). Tappel and co-workers showed that in living cells, protein-bound iron plays a stimulating role in lipoperoxide genesis, a process which could be inhibited by vitamin E (Tappel et al., 1961). Apart from its protein-bound form, iron ions are found in lipid-protein membranes and, in the ferric (Fe^{3+}) state, in ferritin. While the iron bound in ferritin is in a stable state at normal physiologic pH, acidification of the cellular environment can promote iron reduction, setting off a cycle of redox reactions. Further consideration needs to be given to the fact that activation of the enzyme γ -glutamyl transpeptidase (GGT) results in GSH-dependent iron-reduction (Paolicchi et al., 2002). Apparently, GGT catalyzes the first step in the degradation of extracellular GSH by hydrolyzing the γ -glutamyl bond between glutamate and cysteine (Griffith and Meister, 1979). Contrary to expectations from many experiments, in which the anti-oxidant role of glutathione was demonstrated, glutathione might have a pro-oxidant role in the iron-driven redox cycle (Paolicchi et al., 1999).

Oxidation of fatty acids due to free radicals, occurs at the biological membranes even in healthy organisms, which results in membrane alterations such as changes in fluidity and permeability, and in enzyme inactivation when the oxidant load increases (Miquel, 1989). Mitochondrial membranes might be especially vulnerable to those processes, since about 90% of the O_2 used by mammals is processed in the respiratory chain of the inner mitochondrial membrane. Lehninger and Beck (1967) proposed that peroxidative processes are involved in swelling and lysis of mitochondria following injury or during aging

processes, the debris of which is visible as lipofuscin (aging pigment). They further proposed that those peroxidative processes can be inhibited by catalase, an enzyme contained in peroxisomes. Other proposed mechanisms of cellular anti-oxidant defense include those enzymes that remove the radical superoxide. These include glutathione peroxidase which, in the presence of selenium, removes H_2O_2 while oxidizing glutathione (Flohe et al., 1973), and the above mentioned superoxide dismutases (SOD, equations 12 and 13). While oxidative and anti-oxidative processes apparently exist in a homeostatic system in the healthy organism, except for subtle processes of natural aging, the overthrowing of the relative homeostasis by a fundamentally disturbing process such as tissue injury can exceed the anti-oxidant capacity of the cells and result in permanent loss of function and structure.

1.4 The chronic phase of traumatic spinal cord injury

From the morphological aspect, a number of repair processes at the site of injury and in spinal cord segments rostral and caudal to the injury site have been observed during both acute and chronic phases. In many cases, it is difficult to draw an exact line between acute and chronic phase of spinal cord injury. In his book ‘The Cerebral Control of Movement’, Derek Denny-Brown gave a good description of the clinical picture seen and of its morphological basis, and of the transition between the acute and chronic phases. “In the acute spinal animal that is the usual class-room model the only reflex responses other than flexion are the tendon reflex and ‘crossed extension’. Even the most noxious stimulation of skin usually results only in a weak, easily fatigued flexor response, and only in that limb. The emergence of flexion of the limb in response to a nociceptive stimulus to the skin is one of the earliest signs of recovery from spinal shock. The paraplegic limbs then become habitually drawn up in flexed posture (‘paraplegia-in-flexion’). With further recovery the extensors may develop a strong stretch response which then tends to show rapid rhythmical tremor or ‘clonus’. Ischemic damage to the gray matter of the spinal cord, which tends to damage the intermediary and interstitial nerve cells more than those at the periphery of gray matter, and to spare the white matter, leads to selective heightening of the stretch reflex with corresponding impairment of the flexion reflex. Intense spasticity of the extended limbs is then the result of unopposed extensor reflexes.” (Denny-Brown, 1966).

Wound healing processes after tissue injury, regardless of the site of injury, typically fall into three categories that are interdependent and overlapping: inflammation, tissue formation and tissue remodeling (Clark, 1996). The product of wound repair is usually a fibroproliferative rather than regenerative event, with the result of those repair processes

being a 'patch' rather than restoration of structurally and functionally intact tissue (Clark, 1996).

One of the characteristics after spinal cord injury, once the edema has subsided, is the rapid spreading of rostro-caudal cell necrosis, which results in the formation of cystic cavities filled with macrophages and lined by activated astrocytes (Balentine, 1978; Noble and Wrathall, 1985; Bresnahan et al., 1991; Hausmann, 2003). This secondary expansion of the lesion is associated with activation of microglia and influx of peripheral immune cells into the lesion (Stoll et al., 2002). It has been suggested from the results of animal experiments, that there are two types of cavities developing after spinal cord injury (Lagord et al., 2002). In the first type, astrocytes only partially line the walls of the cavities, and the lumen is filled with extracellular matrix proteins and reactive astrocyte processes. In the second type, the walls of the cavity are lined by glia limitans and the lumen is empty. Both types of cavity formation have also been observed in the spinal cords of human patients after injury. Although cavity formation is most commonly observed at the center of the injury site, cyst and syrinx formation can occasionally be found several segments distant from the original site of injury. Extension of syrinx size has been observed several months to years after the injury, even after phases of apparent stability (Hughes, 1988). This implies that degenerative processes must be active long after the primary injury. As a matter of fact, it has been suggested that in human patients cell death by apoptosis continues as late as several years after spinal cord trauma (Johnson et al, 1995). This provides a valid justification to investigate whether compounds with known anti-apoptotic abilities can prevent this late post-traumatic damage.

Type I cavities, filled with collagenous scar tissue, are frequently found in spinal cords of patients who have survived several years after spinal cord trauma (Hughes, 1988). It has been observed that severed nerve endings show indications of sprouting, yet those new nerve endings do not penetrate the neuroglial scar (Hughes, 1984). Although most studies describing regeneration after spinal cord injury have been performed in transection models, sprouting has also been described after spinal cord contusion (Holmes et al., 1998). The description of sprouting by Liu and Chambers was one of the earlier descriptions of plasticity after spinal cord injury in a rat model (Liu and Chambers, 1958). (Goldberger et al., 1993). Sprouting might be a dual sword in the overall complex of circuit re-arrangements after spinal cord injury. On the one hand, sprouting apparently contributes to recovery of excitability of neurons denervated by spinal cord injury (Krassiukov and Weaver, 1996; Li et al., 1996; Saruhashi et al., 1996; Beattie et al., 1993). On the other hand, aberrant sprouting might cause adverse effects such as spasticity and pain, owing to innervation of the wrong target structures (Lindsey et al., 1998; Christensen and Hulsebosch, 1997). In order to attempt functionally successful repair of spinal cord injury, therefore, focus needs to be on two points: 1) Modulation of scar formation in a way that would allow regenerative nerve sprouts to pass through the zone of tissue repair, and 2) target-appropriate guidance of regenerating nerve fibers.

Chapter 2

Potential therapeutic approaches

In 1928, Edgar Douglas Adrian in his book *'The Basis of Sensation'* described the nerve fiber as the conducting unit of the nervous system, consisting of a long thread of protoplasm called the axis cylinder, attached at one end to the nerve cell and usually enclosed in a tubular medullary sheath of a complex fatty substance (Adrian, 1928). Adrian was one of the first to perform conduction studies on healthy nerves (Lucas and Adrian, 1917). Developments in electron microscopy and electrophysiology allowed increasingly detailed studies of anatomical and physiological properties of the nerve cell. Over the years, the pathologic phenomena of spinal cord injury and possible strategies for recovery of function have been studied from a variety of aspects. Those strategies focus on prevention of secondary damage as well as on the possibilities of repair.

2.1 Preventing secondary injury

Unless complete transection of the spinal cord occurs, usually a rim of spared white matter can be found even at the level of injury (Kamencic et al. 2001; Beattie et al., 1997; Basso et al., 1996; Noble and Wrathall, 1985). The results of several studies in models of thoracic spinal cord injury have suggested that the amount of spared tissue highly correlates with recovery of motor function (Kamencic et al., 2001; Basso et al., 1996; Behrmann et al., 1992, Bresnahan et al., 1987). Therefore, preventing secondary damage is synonymous with increasing the potential for better functional recovery. Ischemia and anoxia, inflammatory responses with influx of neutrophils and macrophages, the generation of free radicals and other oxidant species contribute to increase in the volume of post-traumatic damage (Steeves and Tetzlaff, 2000). Ischemia and anoxia result quickly in energy depletion, which is reflected in failure of the $\text{Na}^+ \text{-K}^+ \text{-ATPase}$ and consequent accumulation

of axoplasmic sodium content (Kurihara, 1985; Stys, 1998). At the same time, the number of oxidant species at the site of injury increases significantly (Kurihara, 1985). Therefore, several approaches are conceivable to limit the extent of secondary cell death by apoptosis.

1) Decrease of post-traumatic ischemia and increase of available substrate for energy production. 2) Administration of compounds that decrease apoptotic cell death and 3) Administration of antioxidant and anti-inflammatory compounds.

The benefit of quercetin administration to tissue damaged by ischemia-reperfusion has been studied in animal models of injury to the kidney. For the renal ischemia studies, adult rats were either pre-treated with 50 mg/kg quercetin i.p. 60 minutes prior to ischemia of 45 minutes duration (Kahraman et al., 2003), or quercetin had been administered following 30 minutes of ischemia in the same dose (Inal et al., 2002). Quercetin administration was shown to significantly attenuate increase of enzymes and markers typical for post-ischemic damage, such as myeloperoxidase (MPO) levels, thiobarbituric acid reactive substances (TBARS), TNF-alpha levels (Kahraman et al., 2003). On the other hand, administration of quercetin in the setting of renal ischemia resulted in significantly less decrease in the activity levels of cytoprotective enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase than in untreated animals (Inal et al., 2002). No statistically significant differences between quercetin-treated animals and animals not exposed to ischemic injury could be detected. GSH levels were found to be significantly increased with quercetin administration in one study (Kahraman et al., 2003), while no such significance was found in the other study (Inal et al., 2002). The authors of both studies agree that quercetin reduces oxidative injury caused by renal ischemia. In a model of hepatic ischemia-reperfusion injury in the adult rat, 0.13 mmol/kg quercetin was administered orally

50 minutes prior to injury. Like in the renal ischemia model, protective enzymes such as SOD and glutathione peroxidase were significantly increased with quercetin administration, while the content of reactive oxygen species was significantly reduced (Su et al., 2003). DNA fragmentation after ischemic injury, investigated in the same study, was not found to be significantly altered by quercetin administration. The agreement between the results from these three studies, performed in different organs and testing pre- and post injury treatment, suggest that quercetin might be equally protective in the setting of CNS injury. Acute spinal cord injury, as produced with models of spinal cord compression injury, contains a strong ischemic component during the clip compression phase and with ensuing edema. This seems to be a good model to test quercetin as a neuroprotectant in this setting. A good example of the neuroprotective benefit of quercetin is found in a study by Popovich and colleagues (1999). They demonstrated in an animal model of spinal cord injury that, when hematogenous macrophages were depleted between one and six days after injury, decreased presence of macrophages at the site of injury correlated with improved recovery of motor function. Histological analysis showed significant preservation of myelinated axons and decreased extent of cavitation, which is equivalent with reduction of post-traumatic cell death. Contrary to attempts to reduce secondary damage by administration of specific channel blockers, the focus on inactivation of peripheral macrophages appears to be a more causative approach.

Exact knowledge of the time course of inflammatory responses after spinal cord injury is extremely important in the design of therapeutic strategies. For example, if a single dose of IL-10, a cytokine produced by T-helper cells, macrophages, astrocytes and microglia, which is believed to reduce TNF- α production, was given as early as 30 minutes after spinal

cord contusion injury in rats, anatomical and behavioral outcomes were significantly improved 2 months after injury (Bethea et al., 1999). In the same model, if an additional dose of IL-10 was given 3 days after injury, the protective effect was completely lost. The conclusion from this experiment might be that inflammatory response early after injury is deleterious, while inflammatory response at a later stage is actually protective, being part of the reparative process.

The presence of T-cells is not only essential for the activation of macrophages but also for the mounting of an immune response. A very interesting hypothesis has been based on the observations that there is significant influx of T-cells into the site of spinal cord injury (Sroga et al., 2003) and that the time course of propagated delayed demyelination is very reminiscent of the course of classical auto-immune diseases. The CNS self-antigen myelin basic protein has been made the focus of a number of experiments. It has been shown that injection with syngeneic T cells specific to myelin basic protein resulted in increased accumulation of T cells at the injury site, which was accompanied by decrease of histologically detectable secondary injury and improved electrophysiological parameters (Hirschberg et al., 1998). It appears that modulation of the immune system after spinal cord trauma could be a promising approach to reduction of secondary injury (Schwartz, 2000).

2.2 Bridging the gap – repair of manifest damage

Several promising strategies are currently being explored to repair damage after CNS injury. Those strategies include manipulation of the tissue environment, to make it more conducive to endogenous repair processes, transplantation of autologous or heterologous tissues and implantation of artificial endoprotheses to create guidance channels for regeneration of spinal cord structures.

Just as developing neurons depend on target-derived trophic support, mature CNS neurons depend on neurotrophic support for continuous survival and proper function (Bregman, 2000). In the setting of spinal cord injury, neurotrophic communication between central structures and structures at and caudal to the site of injury might be interrupted. To assure survival and possibly recovery of function, neurotrophic factors can be administered in isolated form or via tissue transplants that genuinely produce them. It has been shown that administration of neurotrophic factors facilitated regrowth of neurons after spinal cord injury into artificial guidance channels or Schwann cell grafts (Xu et al, 1995a and 1995b). It was hoped that transplantation of fetal spinal cord tissue grafts would serve requirements for neurotrophic factor delivery and for creation of a tissue environment permissive for repair at the same time. However, when fetal spinal cord tissue was implanted in the transection site of newborn rats, it was shown that 1) the transplanted tissue can survive in the host environment, 2) the neurons of the transplanted cells are smaller than those of the host tissue and 3) the transplants only appose themselves to the host spinal cord tissue, but do not cross the cellular barrier into the host tissue (Diener and Bregman, 1998).

Since proper, healthy function in the nervous system in many cases depends on the existence of an intact myelin sheath, loss of the myelin sheath due to injury or degenerative

disease results in loss of function. Although some remyelination has been shown to occur spontaneously after SCI (Gledhill and McDonald, 1977; Gledhill et al., 1973), those remyelinated structures are lacking the quality of healthy, undamaged structures, both morphologically and functionally. For example, remyelinated internodes are shorter and thinner than those in healthy subjects (Gledhill and McDonald, 1977; Harrison and McDonald, 1977). Hence, the idea of transplanting cells which might be able to support more effectively the repair of the myelin sheaths. The most promising cells in this context appear to be olfactory ensheathing cells. Olfactory ensheathing cells and their accompanying ensheathing glia are apparently able to traverse both peripheral and central nervous systems, where they contribute to formation of glia limitans and axon remyelination (Doucette, 1984 and 1991; Ramón-Cueto and Valverde, 1995; Franklin et al., 1996; Santos-Benito and Ramon-Cueto, 2003). Li and colleagues were able to demonstrate recovery of function after cervical spinal cord injury in an animal model after transplantation of olfactory ensheathing cells from adult rats (Li et al., 2003).

Appealing for potential therapeutic use in human patients are methods which do not require surgical intervention, thereby significantly reducing the risk of additional iatrogenic injury. In this regard, Akiyama and colleagues demonstrated, in an animal model of contusive spinal cord injury, that intravenous administration of bone marrow cells can contribute to remyelination at the site of injury, with characteristics of both oligodendrocyte and Schwann cell myelination (Akiyama et al., 2002).

In addition to degeneration of the myelin sheath, characteristic atrophic degeneration of neurons is found, as is formation of a glial scar and deposition of extracellular matrix molecules. The release of cytokines and chemokines at the site of injury may render the

tissue environment hostile to repair processes and glial scar formation ensues (Logan et al., 1994; Fitch and Silver, 1997; Steeves and Tetzlaff, 2000). Since there is evidence for both growth-inhibitory and growth-permissive functions of extracellular matrix molecules (McTigue et al., 2000), manipulation of scar formation might be a promising therapeutic approach. Studies by Tetzlaff and colleagues on neurons in the rubrospinal tract have shown that atrophy of neurons can be prevented or even reversed by administration of brain-derived neurotrophic factor (BDNF), which makes even delayed onset of treatment a distinct possibility (Tetzlaff et al., 1994; Kobayashi et al., 1997).

The most likely solution to functional repair after spinal cord injury is a combination of several approaches. A good example supporting this hypothesis is the work of Ianotti and colleagues, who reported on a series of experiments in which the implantation of Matrigel guidance channels at the injury site was tested against additional 1) implantation of Schwann cells, 2) administration of glial cell line-derived neurotrophic factor (GDNF) and 3) a combination of all three modalities (Ianotti et al., 2003). They were able to demonstrate that Matrigel guidance channels alone resulted in only limited axonal growth. With addition of either Schwann cells or GDNF, consistent axonal ingrowth into the guidance channels, containing both myelinated and unmyelinated axons, was observed. The best results in matters of regrowth, myelination and reduction of reactive gliosis were seen with addition of both Schwann cells and GDNF to the guidance channels simultaneously.

Chapter 3

Choice of the Model

In any experiment performed to study mechanisms pertaining to human pathology the choice of an appropriate model is crucial to the validity of the study. Although some aspects of the model might be more important than others, careful consideration must be given to the known variables in the model and to the human pathology modeled. The choice of tests needs to be sensibly weighed against time and space restraints in the model, taking into account possible differences in pathways between species and the limitations of testing functional outcomes in an animal model. Finally, the question should be asked whether an improvement found in an animal model would correspond to functionally valuable improvement in human patients. This is especially true if the treatment requires a high level of effort and compliance on the part of the patient. Failure to consider those aspects will lead to a waste of money, time and effort in both bench research and clinical trials. It also will, quite unnecessarily, disappoint patients and their families, who have put hope and trust in physicians for carrying out the clinical trial.

Acute traumatic spinal cord injury is less variable than traumatic brain injury in terms of pathology, loss and recovery of function, sequelae and extent of recovery. This needs to be taken into consideration when planning clinical trials or laboratory experiments. Although the major advantage of tissue culture experiments or isolated segment studies is an almost universal control over the experimental milieu by the researcher, mechanisms in mammalian organisms are much more complex. Insights about specific pathways may be gained *in vitro*, but information gained from those experiments can not be directly extrapolated to higher organisms. In order to test any specific compound's efficacy to improve recovery of motor function after acute traumatic SCI, it is advisable to use an animal model in which important components, such as the severity of the primary impact and treatment conditions can be

controlled to a certain extent. Such an animal model would eliminate genetic diversity within the strain of the tested species as the major uncontrollable variable. Also, the genetic make-up of the chosen animal species should differ as little as possible from that of humans. This would minimize the chance that genetic differences might cause a disparity in outcome between animal model and human patients. Hence, the animal model chosen should be a mammalian model, where such highly complex mechanisms as those seen in the recovery of motor function are concerned. While a genetic pool strikingly similar to that of humans would make the big apes ideal candidates for pre-clinical trials, the high numbers of subjects required in the early phases of animal experiments, the requirement for specialized facilities and financial restraints speak against their use. Rats, on the other hand, are readily available, affordable and easy to care for even in higher numbers. To make up for the greater genetic differences, pathways of action influenced by the compound tested should be chosen very carefully. If high concordance is found between the pathways influenced by the compound with pathways known in human pathology, there should be a high likelihood that the compound, when beneficial in the animal model, also would have beneficial effects in human patients. For instance, large volumes of secondary tissue loss and cavity-formation after SCI are seen in humans and rats, but not in mice (Kuhn and Wrathall, 1998; Sroga et al., 2003). Since the formation of post-traumatic cavities in the spinal cord will very likely influence functional performance, the lack of concordance between mice and humans gives reason enough to choose the more expensive rat model over a mouse model, where recovery of motor function is investigated. Finally, but not less importantly, the model should allow reproducibility of results between different researchers and between different research centers.

3.1 Small animal models of spinal cord injury

Since animal models provide a unique opportunity to test therapeutic strategies in vivo, they are indispensable components on the way to clinical application. Over the last decades, various models of spinal cord injury have been developed for small animals, namely mice and rats. Clear distinction can be made between models in which the initial continuity of spinal cord elements is largely preserved, as in models of spinal cord contusion or compression, and those where continuity is lost, as in partial and complete transection models. Models of complete transection have the advantage of a greater level of uniformity, as compared to models of contusion and compression, where minor variations in the localization of the injury can cause significant variation in the degree of recovery. Transection makes it somewhat easier to evaluate the effectiveness of interventions with regards to both axonal regeneration and functional recovery (Kwon and Tetzlaff, 2001). A further peculiarity in the setting of transection injury is that, contrary to spinal cord injury caused by contusion or compression, only limited spread of rostro-caudal secondary injury has been observed (Dushart and Schwab, 1994). Spread of injury after transection is expected to occur by Wallerian degeneration (Beattie et al, 2002; Hausmann, 2003). A major difference between transection injury in the animal model and the transected human spinal cord is that the dura has to be opened in order to create the injury in the animal model. Consequently, when edema develops in this setting, the intradural pressure will probably never reach levels as high as in human contusion or compression injuries where the dura mater usually stays intact. The use of partial transection models has at least one clear advantage over either complete transection, spinal cord compression or contusion models:

animals are much less compromised and much easier to care for. Partial transection models have the advantage in that they allow the researcher to selectively injure specific tracts of interest to his field of study, and to use the uninjured side of the same animal as control for his experiment. The major disadvantages with the use of partial transection models are that 1) exactness of transection is not easy to ensure, and 2) functional compensation from the uninjured, contralateral side might be mistaken for functional recovery on the side of injury. Injection of axonal tracers might be required to distinguish between axons that have regenerated and those that have escaped injury in the first place (Kwon and Tetzlaff, 2001). In the clinical setting of acute traumatic spinal cord injury, however, a high percentage of patients do not present with complete transection of the spinal cord, but rather with a blunt injury containing elements of contusion, compression and possibly partial transection (Kakulas, 1984). Only secondary pathomechanisms may result, at a later point, in complete loss of functional spinal cord tissue across the whole cross-sectional surface of the cord at the site of injury (Fehlings and Tator, 1988; Tator and Rowed, 1979). Therefore, compression or contusion models of spinal cord injury more closely simulate the clinical situation seen in the majority of cases. A major difference in the pathology of blunt spinal cord injury, as opposed to transection, is that even with severe injuries, a small peripheral rim of spared axons usually remains (Bresnahan et al., 1987; Basso et al., 1996; Kamencic et al., 2001). This observation made in animal models of spinal cord injury is paralleled by autopsy findings in human patients with neurologically complete spinal cord injury (Hayes and Kakulas, 1997). It has been suggested that those spared axons might be a good target for therapeutic intervention after SCI (Beattie and Bresnahan, 2000; Kamencic et al., 2001).

In 1911, Allen published a historic paper, reporting results from experiments performed with a weight drop model in dogs. Although others had previously performed research on the mechanisms of spinal cord injury in animals, Allen is believed to have been the first to standardize the conditions of the injury (Fehlings and Tator, 1988). Various models for the study of standardized blunt spinal cord trauma have been developed during the 20th century. Presently, the most commonly used animal models of non-transection spinal cord injury are either those of a contusion approach, such as the mechanical NYU impactor or the electromechanical Ohio State University model. Alternatively, timed compression of the spinal cord is used to create the injury. In contusion injuries caused by the weight drop model or electromechanical devices, the site of injury is characterized by the development of a central hemorrhagic necrosis, which spreads both radially and in rostro-caudal direction and later develops into cystic cavities with irregular margins (Bresnahan et al., 1976 and 1991; Guizar-Sahagun et al., 1994; Zhang et al., 1997). The weight-drop models have a number of disadvantages, some of which are due to the method itself. It has been reported that weight drop models produce a considerable variability in clinical outcome and in the pathology at the site of injury (Khan et al., 1985; Khan and Griebel, 1983). Furthermore it was noticed that the weight drop models cause primarily posterior cord compression while, in the clinical situation of human spinal cord trauma, more often the circumferential type of spinal cord compression occurs. Spinal cord compression injury, on the other hand, has been successfully modeled by intraspinal extradural balloon compression (Tarlov, 1957) or by temporary closure of an aneurysm clip around the exposed spinal cord, as in the model developed by Rivlin and Tator (1978).

The decision for either type of injury, transection vs. non-transection model, will be guided by the hypothesis to be tested. However, the decision between several available models representing a similar type of injury might rather reflect the school of thought regarding the mechanism of spinal cord injury to which the researcher belongs. Another interesting point is the choice between wild-type and genetically modified animals as test subjects. Different small animal models with knock-in or knock-out genes have been developed to study isolated pathways and pathology, the simulation of which would have been impossible in wild type animals. We believe, however, that testing of potentially therapeutic compounds should be done in wild type animals, which can be assumed to represent most closely the situation of the average human patient.

3.2 The Rivlin / Tator model

The underlying mechanism most frequently seen in human patients with spinal cord injury is acute compression of the spinal cord by bone following fracture-dislocation or burst fracture of vertebrae (Tator, 1983). Therefore, we chose to use an animal model of acute spinal cord compression injury for studies of functional recovery after acute traumatic SCI.

In 1978, Rivlin and Tator published a paper in which they described their animal model for standardized acute traumatic spinal cord compression injury (Rivlin and Tator, 1978). In this model, the spinal cord of the animal is exposed by laminectomy. Staying extradurally, the blades of an open Kerr-Lougheed aneurysm clip are carefully passed around the exposed spinal cord. At this point, the blades are released quickly to produce acute spinal cord compression injury. The severity of the injury can be varied intentionally through changes in two variables. The closing force between the blades of the clip can be calibrated fairly exactly, by applying springs of different strengths (Dolan and Tator, 1979). Secondly, variation of the duration of clip-closure around the spinal cord will produce injuries at different levels of severity (Dolan et al., 1980). This model is simple and reproducible in small rodents, and it allows the researcher to vary the severity of the injury produced (Dolan et al, 1980). The injuries generated with the clip compression model were found to be much more consistent with respect to clinical recovery than injuries produced by the weight drop method (Khan and Griebel, 1983).

Contrary to many other models of acute spinal cord injury, animals injured with the clip compression model are completely paraplegic after clip application. Both hind limbs are flaccid, and no withdrawal response is elicited by pinching the hind paws. There is no

spontaneous micturition, and bladders need to be manually expressed three times daily until bladder function is recovered. The latter can be expected between two and three weeks after injury, although some animals do not recover spontaneous bladder function at all. In some studies, recovery of urinary tract function has actually been used as measure for functional recovery after spinal cord injury (Pikov et al., 1998; Chancellor et al., 1994).

3.2.1 Clinical validity of the model

Usually, the researcher has only limited influence on external restrictions to his research, such as limitations of time and space, available personnel and financial support. Yet, the one restraint the investigator will put on herself, the choice of experimental design, will make the greatest impact on the success of her work. Since the experimental design greatly determines the limitations of the planned experiments, it is of crucial importance for the clinical validity of results.

3.2.1.1 Location of injury

Statistically, 55% of all human traumatic spinal cord injuries are reported to occur at cervical level. The remaining 45% percent are almost evenly divided between thoracic (T1-T11), thoracolumbar (T11-L2) and lumbosacral (L2-S5) locations (Sekhon and Fehlings, 2001). Yet, one can not infer from those statistics that a clinically valid animal model should be preferably one of quadriplegia.

The first point to consider is the feasibility of experiments using quadriplegic animals. An animal model of quadriplegia would create several significant problems with regard to animal care. According to our observations, caged paraplegic rodents compensate

surprisingly well for the loss of hind limb function. While the animals are significantly limited in their perambulation, basic daily functions such as feeding and grooming are not impaired. Quadriplegic animals would be largely immobilized and unable to perform either of the required daily functions. Not only would this significantly reduce the comfort level for the animals, but additional disease caused by lack of the usual body hygiene could be expected.

The second aspect pertains to the anatomical and pathophysiological differences between injuries accidentally occurring or and those intentionally created at various spinal cord levels. Given the observation that spinal cord injury in our model results in proportionally more destruction of gray matter than white matter, the morphological distribution of gray and white matter throughout the spinal cord should be of foremost concern. It is well known that the distribution pattern of gray matter changes considerably in cervico-caudal direction. The size of gray matter is proportional to the amount of skeletal muscles innervated at any given segment. Consequently, we see a greater amount of gray matter in those segments which innervate the muscles of the upper and lower limbs, i.e. in cervical and lumbar segments. The increased amount of neurons and neuroglia in the gray matter at those segments is reflected in the size of the cervical and lumbar enlargements. Thus, proportionally more gray matter is present in cervical and lumbar spinal cord regions than at thoracic level. Therefore, one could speculate that, since neuronal cell bodies and neuroglia are apparently more sensitive to the consequences of acute spinal cord trauma than axons in the white matter, the morphological extent of injury might be somewhat larger if clip compression with the same force and for the same period of time were to be applied to cervical or lumbar regions of the spinal cord.

If an animal model with a lesion caused at thoracic level is used, the segmental circuits and motoneurons necessary for locomotion are spared. This fact makes the analysis of motor recovery after injury somewhat more complex, since the induction of local pattern generators alone can allow for a certain degree of spontaneous recovery of motor function even without treatment (Beattie and Bresnahan, 2000; Schültke, unpublished data). From the above considerations, the question arises whether, in order to simulate injury in a spinal cord segment with a high proportion of gray matter yet avoid working with quadriplegic animals, it would be advantageous or necessary to inflict a lumbar rather than thoracic injury. A strong reason against this approach is the fact that, given the anatomic differences between thoracic and lumbar vertebrae in the rat, the surgical approach to the lumbar spinal cord would be considerably more traumatic to the animal. Since it now has been acknowledged that the presence of local pattern generators is not specific or confined to non-primates, but that they also exist in humans (Barbeau et al., 1999; Marder and Bucher, 2001), we found it acceptable that part of the recovery induced with administration of a potentially therapeutic compound might be due to manipulation of local circuits rather than to recovery of descending pathways.

Various investigations show evidence that function of the immune system is suppressed in patients with complete spinal cord injury at higher cord levels (Campagnolo et al., 1997 and 1994; Nash, 1994, Cruse et al., 1993). Stress responses, characterized by acute or chronic adrenergic overstimulation, have been associated with immune dysfunction (Madden et al., 1989; Madden and Livnat, 1991; Cruse et al., 1993). Explanations for this phenomenon include autonomic dysfunction caused by direct injury to structures of the autonomic nervous system or autonomic dysreflexia, where injury is above the level of sympathetic adrenergic outflow (T 6). Imprudent diet and physical inactivity of patients after SCI can result in further suppression of the immune system (Nash, 2000 and 1994). There is clinical evidence that patients who sustain complete spinal cord injury above the level of T 10 experience much more alterations in immune function than those with injury below T 10 (Campagnolo, 2000). Although, to our knowledge, no description linking immune function to the level of injury exists for animal models, we took the possibility into account. To allow applicability in a high number of clinical situations, we decided to use an animal model of complete spinal cord injury rostral to level T 10. To avoid much of the dysreflexia caused by injury at or above spinal cord level T 6, we chose spinal cord level T 7 for our experiments.

3.3.1.2 Injury Mechanics

The primary injury in our animal model, inflicted by temporary closure of the aneurysm clip around the entire spinal cord, includes elements of contusion and compression. Contusion is caused by the shanks of the clip impacting on the spinal cord tissue at the beginning of the closing phase. The mechanism of cord compression between the closed shanks is obvious, and severity of the injury depends on the duration of clip closure (Dolan et al, 1980). Contusion results in immediate mechanical injury with disruption of structure and hemorrhages, and compression results in temporary tissue displacement and ischemia. This injury mechanism closely simulates the clinical situation of a patient suffering a spinal trauma, in which vertebral fragments are forcefully propelled into the spinal canal (contusion) and consequently lead to compression of the spinal cord.

The extent of the ensuing secondary injury in both human SCI and in our animal model is determined by several factors, including the force of injury, the timing of ischemia and reperfusion as determined by the duration of compression, and by the ability of the organism for repair. While the exact force or duration of the traumatic impact on the spinal cord is rarely known in human SCI, these parameters are relatively well controlled in our animal model. Since the closing force of the clip is regularly checked and re-calibrated if necessary, the variable portion of the injury in our model lies almost entirely in the anatomical variability of the individual animals. As in human patients, factors such as the width of the spinal canal, the angles of spinal root entry and exit or the pattern of blood supply of spinal cord segments vary among individuals and might influence the extent of damage created by primary and secondary injury. From the aspect of the surgical procedure, the presence of a major blood vessel in the planned field of surgery would necessitate clip

application at a slightly more rostral or caudal level than planned. However, in the thoracic region, a minor variation in the level of spinal cord injury would not be expected to result in significant differences in the injury pattern. A circumstance that might influence the severity of the impact, however, would occur if the surgeon were unaware that a small bone fragment created by the laminectomy is caught between the blades of the aneurysm clip. In this case, several scenarios are possible. If the bone fragment were caught between the branches of the clip where no spinal cord tissue is interspersed, the clip blades might be prevented from closing completely, thereby decreasing the force of injury. A decreased force of injury would be detectable clinically only if the injury force was lessened to a degree where the procedure resulted in incomplete paraplegia. In a second scenario, if the bone fragment were caught between the branches of the clip and spinal cord tissue, an unintended additional local tissue trauma will be created, and the overall injury is aggravated. Overall, the variability between injuries created in our animal model is considerably less than that observed in human spinal cord injury. This circumstance allows, given that an adequate number of animals are tested in therapeutic and control groups, a realistic assessment whether a tested compound improves function after SCI or not.

Acute traumatic injury to the spinal cord is usually followed by edematous swelling in both human SCI and in the animal model. Since the dura mater and the bony elements forming the spinal canal limit expansion, edema formation can lead to compression of neural structures and occlusion of microvasculature. Occlusion of the microvasculature may result in longer-lasting ischemia, thereby leading to an accumulation of metabolic products in the spinal cord tissue which in turn aggravates the edema. There is one obvious difference in the architecture of the injury site between our animal model and the pathology in those patients

who did not require surgery after spinal trauma. While, in the cases of the latter patients, the bony components of the spinal canal are still present, our model requires laminectomy over the site of injury in all animals. However, we assume that the elasticity of the dura mater, contrary to the soft spinal cord tissue, would require an extremely high intradural pressure in order to be deformed and expanded from the inside. We therefore believe that those differences in the architecture of the injury sites should not have any significant influence on the functional outcome, as long as the dura mater at the site of injury in the animal model remains intact. Animals in which injury to the dura mater has been observed were excluded from our analysis.

Chapter 4

Choice of the compound

4.1 The ideal compound – theoretical requirements

Thought should be given to a number of considerations, before any potentially beneficial compound is tested in vivo. 1) Adverse effects: The most prominent question is naturally whether any significant adverse effects from both the drug itself, and from the route of planned application need to be considered. Ideally, the compound should have been previously tested in in-vivo situations for other applications. This would have resulted in some previous assessment of possible adverse reactions to be expected with use of the compound. If adverse reactions were known, the next step would be to answer the question whether the expected benefit outweighs the possible risk of adverse reactions. Even if no adverse reactions have been observed or described with prior use in a different setting, we still should not forget to observe closely, because the pathological setting created in our model might tax the natural defense mechanisms of the organism in different ways than in models previously used.

2) Pharmacokinetics: In order to make a good estimate with regard to an effective dose, the following pharmacokinetic data should be considered, if available from literature or preliminary experiments: Absorption, to determine possible routes of administration. While, for the animal experiment, the preferred route of administration to assure standardized dosing obviously would be intraperitoneally or subcutaneously, for a later transfer to clinical trials, the option for oral administration might significantly increase acceptability and compliance from the patients' side. Peak intervals and the compound's half-life should be known to significantly reduce the estimation of dose-interval scheduling. Since it is known that, with human patients, compliance increases the less often a drug has to be administered, we would prefer a compound with a half-life of 12 hr or longer. To know the main route of excretion might not seem of major importance in the animal experiment, where functional variability between animals tested is usually limited by standardized weight and age. If human clinical trials are anticipated, however, to know the route of excretion becomes of the utmost importance, to prevent potentially harmful effects from unexpected build-up of drug levels due to partial or complete failure of the eliminating organ system. In this context, it would be most useful to obtain knowledge about LD₅₀ of the compound.

3) Pathways influenced by the compound. Ideally, the hypothesis stating why we believe that our compound should be beneficial in a specific setting, should be based on results from experiments in other model systems. This, compared with an analysis of major pathways governing development of pathology in our model system, should give a good idea about the therapeutic effect we can expect. None of the pathomechanisms causing secondary injury after SCI, such as ischemia, edema, inflammation and lipid membrane peroxidation, has been singled out as the most prominent contributing factor. Therefore, it should be worthwhile to test a compound that has the ability to interfere with more than one of those pathways. Although it might prove more difficult to establish the exact mechanism of action for such a compound, as compared to compounds where action is apparently limited to interference with one single pathway, we feel that a compound's capacity to address several key mechanisms in a balanced manner could improve the therapeutic potential. The ideal compound, therefore, should have a minimum of adverse effects, a half-life of 12 – 24 hr, a relatively high LD₅₀ and an option for oral administration.

4.2 Polyphenolic flavonoids

The aim of this chapter is to illustrate that polyphenolic flavonoids in general, and quercetin specifically, should be well suited for therapy of patients with CNS injury.

Since the emergence of the concept of secondary injury after acute CNS trauma, various compounds have been studied with the hope of minimizing the extent of this secondary damage. Polyphenolic flavonoids have been investigated in a number of model systems, both in vitro and in vivo. Many flavonoids were found to have anti-inflammatory, antioxidant and anti-edematous capacities. Some flavonoids, such as quercetin, are also excellent iron chelators. Considering the fact that inflammatory processes, oxidative stress and edema formation as well as intraparenchymatous hemorrhage are hallmarks of acute CNS trauma, the combination of anti-inflammatory, antioxidant and anti-edematous capacities with its ability to chelate iron should make quercetin a promising compound in the therapy of patients with acute CNS injury. While many of the compounds tested in the setting of CNS trauma are synthetic in origin, polyphenolic flavonoids are commonly found in fruits and vegetables. Flavonoids can not be synthesized by the human body, therefore the only available source are dietary components. Based on dry weight, the highest anti-oxidant contents are found in kale, blackberries, blueberries, cranberries, raspberries and strawberries, and in spinach (Prior and Cao, 2000). Flavonoids are three-ring structures (Fig. 4.1), which can be divided into groups according to substitutes at certain positions, which determine the capacities and the mechanism of metabolic degradation of the flavonoid molecule.

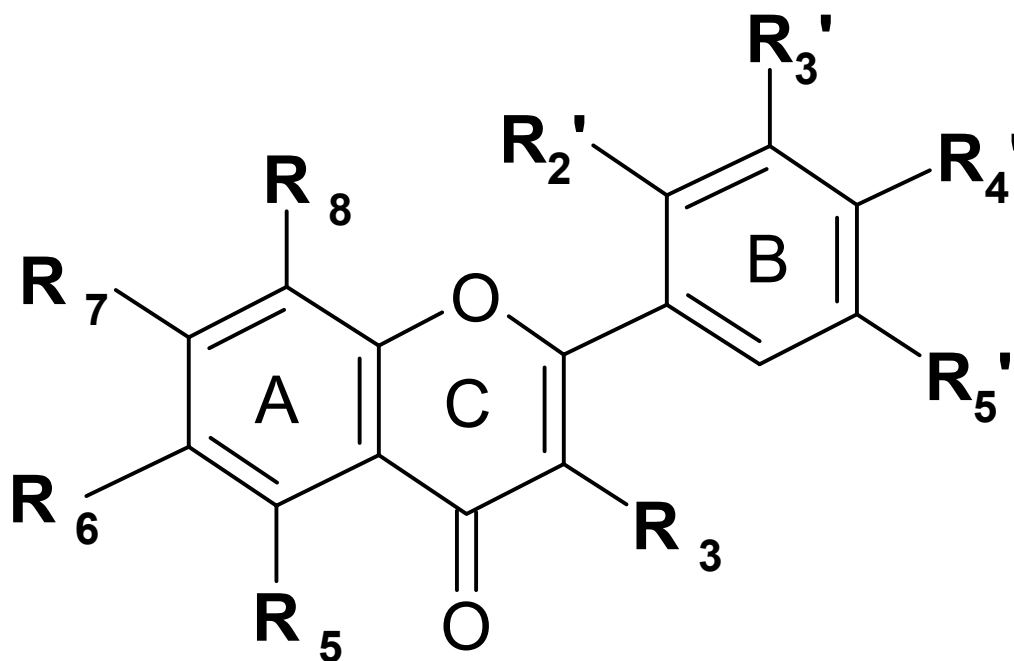


Figure 4.1: General structure of polyphenolic flavonoids.

R may or may not be substituted in individual flavonoids. For most flavonoids, a characteristic number of R is substituted by an OH-group.

There are six major subclasses, which include the flavones, flavonols, flavanones, catechins, anthocyanidins and isoflavones (Ross and Kasum, 2002). The presence of an oxy-group at position 4 in the B ring (as seen in the catechol group, as in quercetin, or pyrogallol group, as in myricetin) and a double bond between carbon atoms 2 and 3 of the C ring

increase the antioxidant capacity, and the presence of a hydroxyl group at position 3 of the C ring appears to be critical for anti-inflammatory activity (Theobarides et al, 2001). The properties of flavonoids have been investigated in various model systems, both in vitro and in vivo.

As mentioned earlier, key pathological mechanisms contributing to increase of secondary injury volume after acute traumatic spinal cord injury include ischemia and vascular dysregulation and ischemia, edema formation, inflammatory processes, lipid membrane peroxidation and contusional hemorrhage (Hall; 1986; Barut, 1993; Tator, 1995; Tator and Koyanagi, 1997; Carlson, 1998; Schnell, 1999; Tator, 2002). While the volume effect of hemorrhages causes further mechanical damage and ischemia, disintegration products of hemoglobin also contribute to increased damage by lipid membrane peroxidation (Matz, 2000; Gaetani, 1998). Polyphenolic flavonoids have been tested in various systems modeling most of those pathological processes.

1) Inflammation – polyphenolic flavonoids act as anti-inflammatories

Polyphenolic flavonoids have been tested in in-vivo models of acute and chronic inflammation. Testing anti-inflammatory activity in the setting of chronic inflammation, a number of flavonoids were shown to inhibit granuloma formation in a model of cotton pellet-induced granuloma formation (Pelzer et al., 1998). Contrary to the antioxidant capacity, this inhibitory effect appeared to be independent of the presence of a double bond between C2 and C3, and C3 conjugation with sugar does not alter the intensity of activity. The presence of a catechol or guaiacol-like B-ring appeared to increase anti-inflammatory activity in this model of chronic inflammation. As a model for acute inflammatory processes, carageenin-

induced paw edema in rodents is commonly used, with reduction in the extent of paw edema as indicator of therapeutic success. Significant reduction of paw edema was seen with several flavonoids, to varying extent (Pelzer et al., 1998). No structure-activity relationship was deduced from those experiments. Possibly, the anti-inflammatory capacity of flavonoids is due to their ability to reduce immobilization and degranulation of leukocytes during the post-traumatic inflammation and / or the ability to deactivate reactive species produced by stimulated neutrophils (Pincemail et al., 1988; Friesenecker et al., 1996).

Oxidative tissue injury describes a setting in which tissue components are modified by reaction with free radicals and other oxidant species, such as hypochlorous acid, resulting in temporary or permanent functional deficit. One of the prominent capacities of polyphenolic flavonoids is their capacity to act as anti-oxidants (Hall, 1986; Barut, 1993; Saija, 1995; Cotelle, 1996; Huk, 1998; Sugihara, 1999). It is believed that anti-oxidant activity is conveyed through inhibition of pro-inflammatory enzymes as well as through chemical reaction of the flavonoids with free radicals (de Groot and Rauen, 1998). Inhibition of enzyme activity by flavonoids has been reported for cyclooxygenase and lipoxygenase (Laughton et al., 1991; Hoult et al., 1994), monooxygenase (Siess et al., 1995), protein kinases (Cushman et al., 1991) and mitochondrial NADH-oxidase (Hodnick et al., 1994). It has been suggested that the ability of flavonoids to inhibit cyclooxygenase and 5-lipoxygenase in the metabolism of arachidonic acid may contribute to their ability to suppress inflammatory processes. However, Sobottka and colleagues did not observe any correlation between the tested flavonoids' ability to reduce paw edema and inhibition of these enzymes (Sobottka et al., 2000).

2) Oxidative stress – polyphenolic flavonoids act as antioxidants

Polyphenolic flavonoids are believed to act as antioxidants either by donation of a hydrogen atom or an electron, which means that the flavonoid itself is being oxidized (Shahidi and Wanasundara, 1992). The mechanisms of oxidation seem to vary with the oxidizing agent. Flavonoids can stabilize reactive oxygen species by reacting with the reactive component of the radical (Nijveldt et al., 2001).



R^{\bullet} describes a free radical and O^{\bullet} denotes a reactive oxygen species.

From experiments done by Jovanovic and colleagues it appears that oxidation of flavonoids with a catechol structure will take place exclusively in the B-ring (Jovanovic et al., 1996).

3) Iron ions increase oxidative injury – some flavonoids are good iron chelators

One of the consequences of CNS trauma is the presence of intraparenchymatous hemorrhage. Caused by the gradual disintegration of hemoglobin, the content of free iron ions in the tissue increases. Iron ions appear to catalyze oxidative stress reactions that result in lipid membrane peroxidation (Fig. 4.2). Hence, iron chelation might limit the potential secondary damage otherwise caused by the post-traumatic presence of free iron ions.

Many studies to determine the anti-oxidant capacity of flavonoids have been performed in various in-vitro models. This may often lead to seemingly contradictory results, with results depending on the oxidant used. As pointed out by Prior and Cao (2000), the free radical source in the experimental set-up is very important and direct comparisons

between systems using different free radical sources should be avoided. A good example is the controversy whether iron chelation plays a major role in the antioxidant activity of flavonoids. Iron ions accumulated in tissues can catalyze the formation of hydroxyl radicals (\bullet HO) from hydrogen peroxide (H_2O_2) and superoxide free radical ($\text{O}_2^{\bullet-}$), as described by the Fenton reaction (Gutteridge et al., 1981). H_2O_2 and $\text{O}_2^{\bullet-}$ are generated by neutrophils at the site of injury.

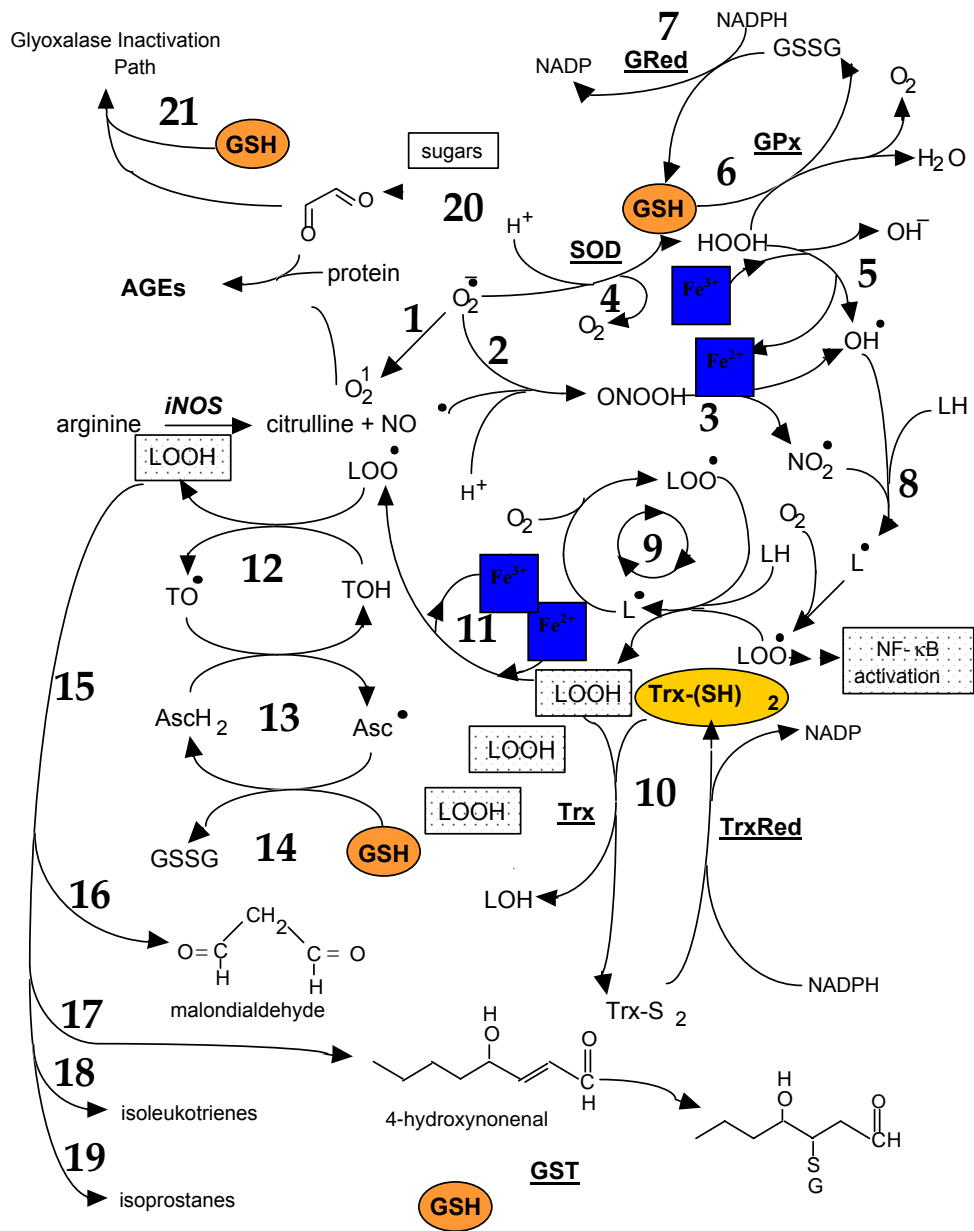


FIGURE 4.2: Overview of some of the pathways involved in production of free radicals and lipid membrane peroxidation. Note the involvement of ferrous (Fe^{+2}) and ferric (Fe^{+3}) iron ions.
 from: Juurlink, 2001

From several in-vitro studies it appears that iron-chelation is one of the mechanisms through which flavonoids act as anti-oxidants, protecting against reactions involving free radicals and iron ions (Morel et al. 1993 and 1994; Ferrali et al., 1997; Cheng and Breen, 2000).

Van Acker and colleagues argued that the results of their experiments indicated that iron chelation played no major role in the antioxidant activity of flavonoids in their model system of microsomal lipid membrane peroxidation. However, they allowed that their experimental set-up might simply not have been suitable to detect the contribution of iron chelation to antioxidant action (van Acker et al., 1998). Sestili and colleagues were also investigating whether iron chelation played any significant role in the antioxidant activity of quercetin. They chose a model of DNA cleavage and cytotoxicity induced by tert-butylhydroperoxide. In this model system, DNA cleavage can be abolished by iron chelators, but not by antioxidants which act without iron chelation (Coleman et al., 1989; Latour et al., 1995; Guidarelli et al., 1997). This is contrary to model systems which use H₂O₂ to induce cell death, where cell death can be decreased by action of both iron chelators and antioxidants without iron-chelating action (Coleman et al., 1989; Guidarelli et al., 1997). Sestili and colleagues found clear evidence that iron chelation is indeed an important mechanism in the antioxidant action by the flavonoid quercetin (Sestili et al., 1998). It has been suggested that the use of naturally occurring metal chelators should be preferable over synthetic chelators, because the former appear to be associated with less adverse effects (Aruoma, 1996).

4.3 Quercetin

Availability / sources: The flavonol quercetin, is one of the better-investigated flavonoids. Significant amounts of dietary quercetin can be found in cranberries, olives, apple skin, broccoli, onions, red wine, green tea and in the propolis of bee hives (Herman, 1976; Havsteen, 1983; Rice-Evans, 1996; Hollman, 1996 and 1995). It was also shown to be an important active ingredient of Ginkgo biloba (Kleijnen and Knipschild, 1992). Just like other members of the flavonoid family, quercetin scavenges free radicals (Huk, 1998), in an anti-oxidative fashion (Cotelle, 1996; Saija, 1995; Sugihara, 1999) and anti-inflammatory (Ferrandiz, 1991; Middleton and Kandaswami, 1992; Pelzer, 1998, Middleton et al., 2000) and anti-edematous (Pelzer, 1998; Sobottka, 2000).

Toxicity: Toxicity of quercetin has been assessed both in vitro and in vivo. Sestili and colleagues found that concentrations of up to 30 μM were not cytotoxic or causing any DNA damage in an in-vitro model of DNA cleavage and cytotoxicity induced by tert-butylhydroxide (Sestili et al., 1998). Nguomo and Jones performed a cytotoxicity study on ovary cells from Chinese hamster, mouse fibroblasts and cultures from normal rat kidney cells. They determined the molecular concentration at which cell growth was inhibited by 50% (IC 50) after 48 hr incubation with quercetin was 80 μM (Ngomuo and Jones, 1996). Khaled and colleagues administered single doses of up to 56 mg/kg to adult rats, either intravenously or orally, without observing any adverse effects within the 24 hr observation period (Khaled et al., 2003). In a toxicity study of human patients who received quercetin, it was shown that doses of 70 mg/kg quercetin can be safely administered as single i.v. bolus, resulting in serum concentrations up to 400 μM immediately after injection (Ferry et al.,

1996). In this study, doses were administered as single bolus intravenously, in 3 week intervals. Doses were increased stepwise from 60 mg/m² to 1700 mg/m². Dose-limiting nephrotoxicity was encountered only at 1700 mg/m².

Absorption and elimination: If recovered from dietary sources, flavonoids are usually attached to sugar residues (Ross and Kasum, 2002). The presence of sugar residues alters the physico-chemical properties of the molecule and therefore their ability to cross membranes and enter cells (Hollman et al., 1999). While aglycones can be absorbed directly from the stomach and then secreted in bile and urine, glycosides have to be enzymatically modified prior to absorption (Piskula, 2000; Crespy et al., 2002; Shimoi et al., 2003) (Fig. 4.3). It has been shown that both quercetin and rutin can be absorbed in all segments of the intestine (Su et al., 2002). Dietary polyphenolic flavonoid glycosides are substrates for β -glucosidases, UDP-glucuronyltransferases or catechol-O-methyltransferases in the small intestine, and for several phase I and phase II enzymes in the liver (Scheline, 1991a; Rice-Evans et al. 2000; Scalbert and Williamson, 2000). Apparently, there are two different mechanisms for uptake of flavonoids across the small intestine. Flavonoids might be deglycosylated on the luminal side by lactase phlorizin hydrolase and subsequently diffuse into the intestinal cells as aglycones (Day et al., 2000; Sesink et al., 2002). On the other hand, the results of several studies suggest that quercetin glycosides interact with a glucose carrier (SGLT1), which facilitates uptake across the brush border membrane of the small intestine (Gee et al., 1998 and 2000; Ader et al., 2001; Wolfram et al., 2002). However, in the mesenteric blood taken from intestinal veins, only conjugated forms of flavonoids were found, regardless whether glucosides or aglycones were administered (Crespy et al., 2001). Furthermore, flavonoids are

metabolized by the colonic microflora, where the breakup of the flavan ring results in much more simply structured phenolic compounds (Scheline, 1991b; Li et al., 2000). As indicated by the activity of endogenous β -glucosidases in vitro, deglycosylation appears to be an early step in flavonoid metabolism in both humans and rats (Day et al., 1998; Ioku et al., 1998). Site of deglycosylation and mode of transport across the intestinal cells depends largely on the nature of the flavonoid aglycone moiety, and on the structure and position of the attached sugar. The latter might be explained with the specificity of residential β -glucosidases and other locally available enzymes (Day et al., 1998 and 2002).

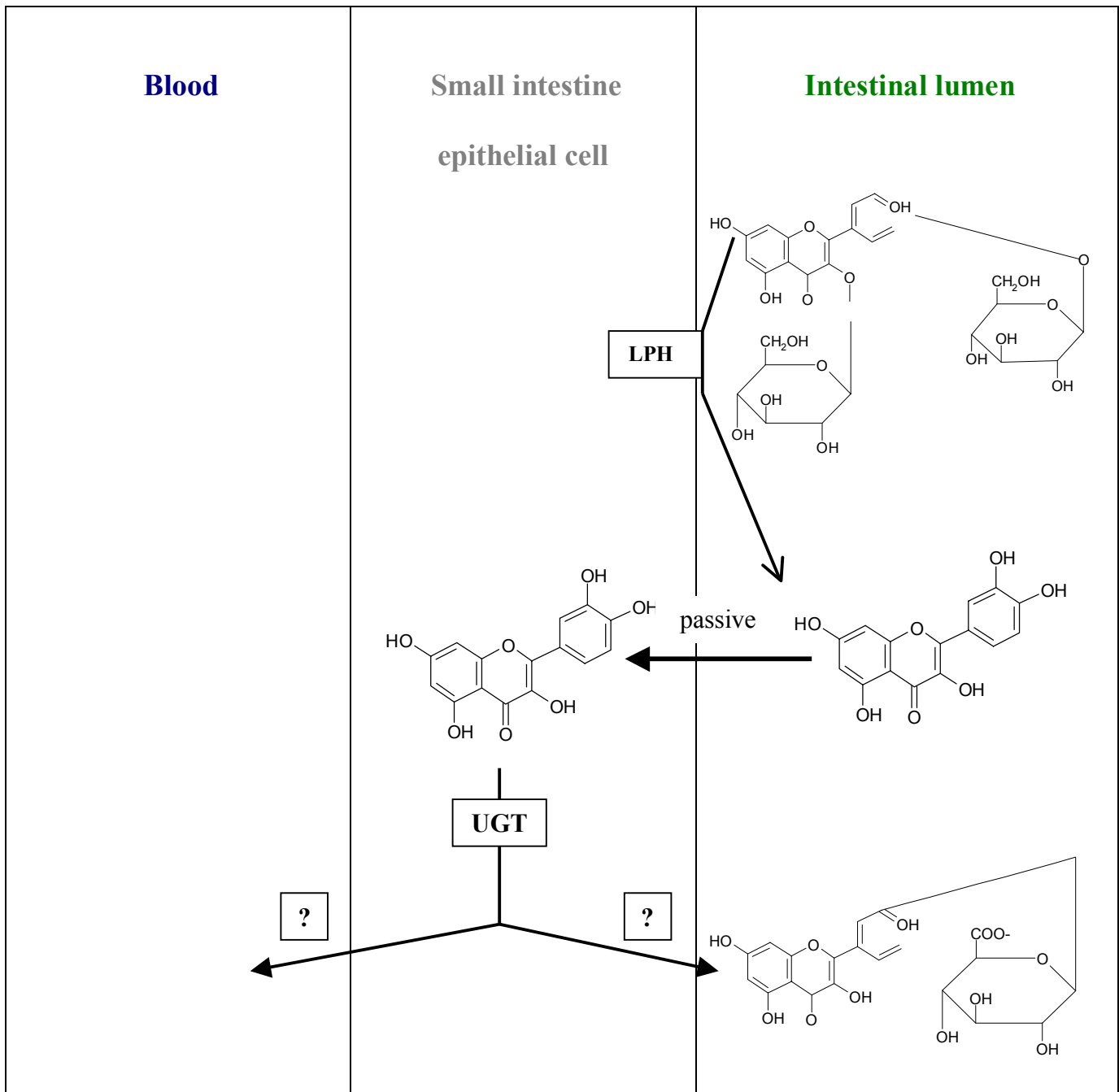


FIGURE 4.3: Possible mechanism of quercetin absorption following oral administration.

LPH: Lactate phlorizin hydrolase; UGT: UDP-glucuronyltransferase

after: Petri et al., 2003

Walle and colleagues studied absorption and elimination of the radioactively marked quercetin aglycone (quercetin dihydrate, Sigma) in human volunteers after both oral and intravenous administration (Walle et al. 2001). As expected from previous studies, there was no unchanged quercetin present after oral administration (Ferry et al., 1996; Walle et al., 2000; Wittig et al., 2001). Terminal elimination half-lives of the aglycone were found between 20 and 72 hr, varying between 31 and 64 hr after oral administration and between 20 and 72 hr after i.v. administration, resulting in half-life means of 41 hr and 40 hr, respectively. Thus, no statistically significant differences in the half-lives were found between oral and intravenous administration of the compound. It should be noted that the half-lives determined in this study are longer than reported in earlier studies reported by other groups (Hollmann et al., 1997; Erlund et al., 2000). This might be due to differences in the experimental set-up. Those studies reported half-lives of 15-28 hr, but targeted quercetin conjugates rather than including further breakdown products. Since metabolic products of quercetin themselves have been shown to possess antioxidant, anti-inflammatory and anti-edematous capacities, Walle's study, taking into account metabolic breakdown products, is doubtless of practical relevance. The results from Walle's study suggest that the seemingly prolonged half-lives of quercetin metabolic products could be due to enterohepatic recirculation, especially after oral administration, as evidenced by a distinct second peak of plasma radioactivity around 8 hr after administration.

Significant differences were found, however, between oral and i.v. administration with regard to recovery of quercetin or its metabolic products (Fig. 4.4). While, after intravenous administration of quercetin dihydrate, on average 21.3% were recovered in urine and 3.2% in feces, only 4.6% were recovered in urine and 1.9% in feces after oral

administration. It is noticeable that, in the group of patients who received quercetin dihydrate orally, the percentage of recovery from feces varied between 0.2 and 4.6%. This might be due to the fact that absorption from the gastrointestinal tract is somewhat slow and irregular, due to its low solubility in water and its slow dissolution rate (Lauro et al., 2002). Another explanation could be differences in the intestinal microflora of the test individuals, as it has been reported that no aglycones were recovered in feces from rats lacking the necessary microflora (Griffiths and Barrow, 1972). As flavonoids and their metabolites are modified by the colonic flora, they in turn modify the composition of the intestinal microflora (Blaut et al., 2003).

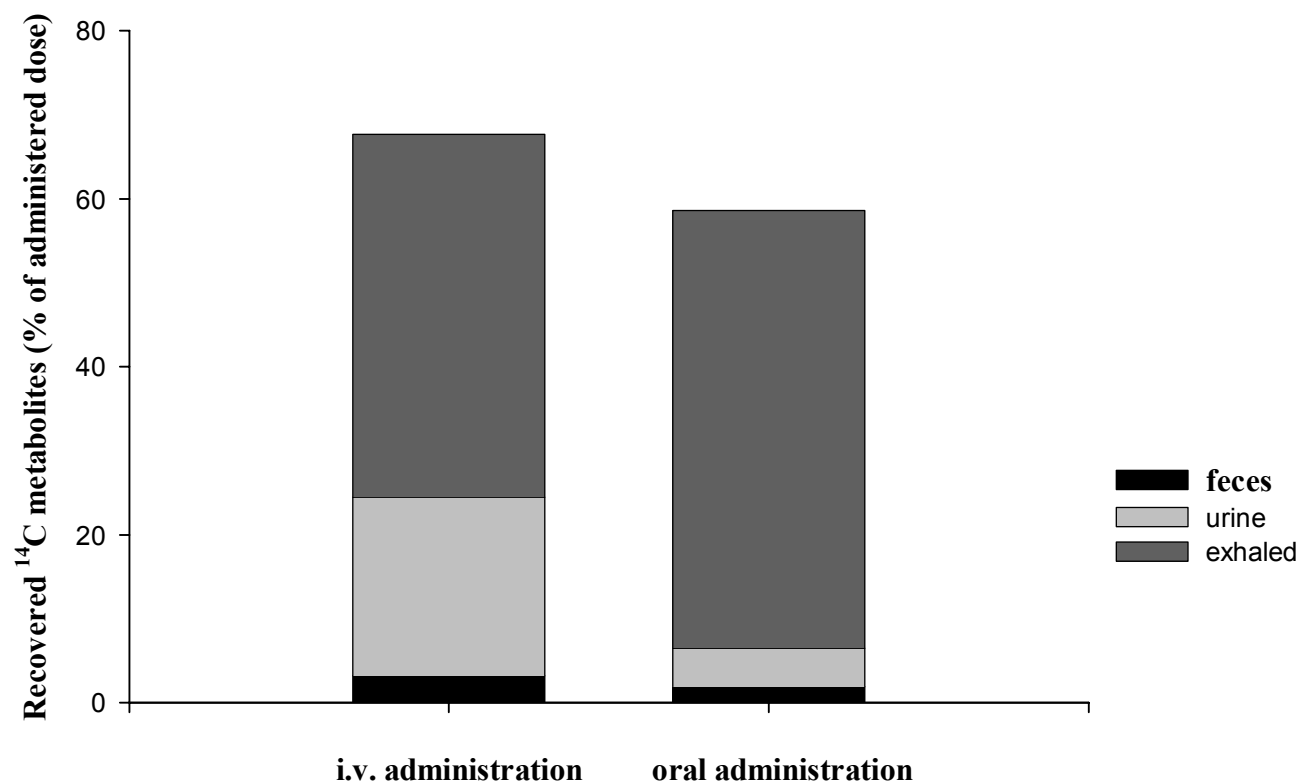


FIGURE 4.4: Excretion of ^{14}C -containing metabolic products of quercetin after single bolus administration (i.v.: 0.3 mg = 1 μmol , oral: 100 mg = 330 μmol).

A higher percentage is excreted with the feces after i.v. than after oral administration. Urinary excretion of quercetin breakdown products is more commonly observed after i.v. administration than after oral administration of the compound.

(after Walle et al. 2001)

With both routes of administration, on average about half of the quercetin appears to be metabolized and exhaled as CO₂, as demonstrated by detection of the radioactive marker (Fig. 4.5). However, while the size of the exhaled fraction varied between 41.8 and 63.9% after oral administration, the variation between 23 and 81% after intravenous administration was much more pronounced. Besides the fact that the power of this part of the study was low (n = 3) the variation seen might be due to differences in the enzymatic activity between different individuals. Observations made in an earlier study, where rat gut was incubated with radiactively marked quercetin produced large amounts of ¹⁴CO₂ (Ueno et al., 1983), Walle and colleagues concluded that the ¹⁴CO₂ recovered in their study was most likely produced by breakdown of quercetin in the intestine.

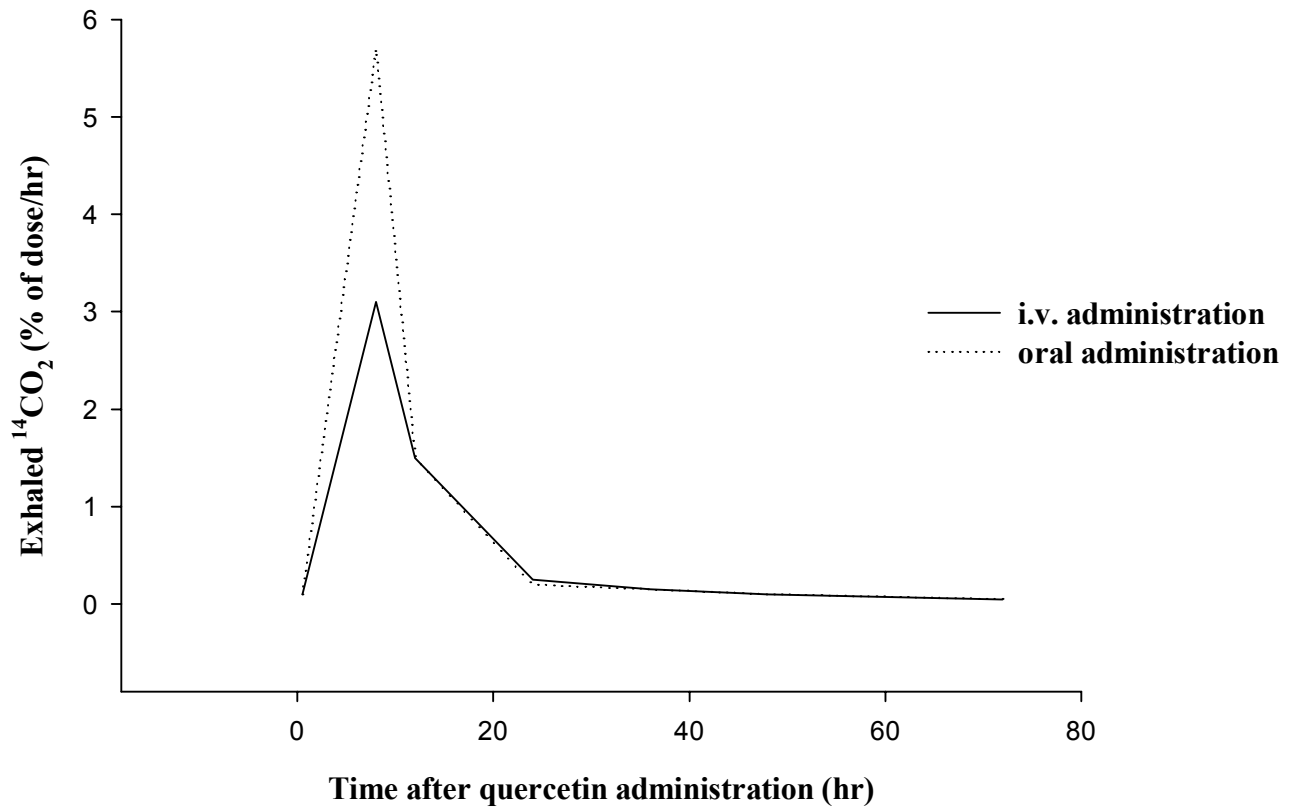


FIGURE 4.5: Exhalation of ¹⁴CO₂ after oral and i.v. administration of quercetin in human volunteers. With both routes of administration, on average about half of the quercetin appears to be metabolized and exhaled as CO₂, as demonstrated by detection of the radioactive marker.

(after Walle et al., 2001)

It has been shown that only between one and two thirds of the administered quercetin doses were absorbed by passage through the stomach or small intestine (Crespy et al., 2002 and 2003).

Attenuation of ischemia. Huk and colleagues demonstrated in an animal model of hindlimb ischemia that, in the presence of quercetin, tissue levels of nitric oxide increased and superoxide levels decreased (Huk et al. 1998). This effect was observed up to concentrations of 30 $\mu\text{mol/l}$. With higher concentrations, no significant beneficial effect was observed. It was demonstrated that quercetin directly scavenged superoxide anions (Sichel et al., 1991; Huk et al., 1998). Quercetin is also known to be a protein kinase C inhibitor (Blackburn et al., 1987). Since constitutive nitric oxide synthetase (cNOS), the enzyme through which endothelial nitric oxide production is regulated, is usually turned off by phosphorylation of one of its serine residues shortly after activation (Sessa, 1994), it is suggested that quercetin prolongs cNOS activity and thereby nitric oxide production. Nitric oxide is a potent vasodilator (Palmer et al., 1987), while superoxide is a potent vasoconstrictor, the latter being effected by the superoxide's ability to scavenge nitric oxide (Consentino et al., 1994). By tipping the balance between vasoconstrictor and vasodilator action in the latter direction, the net effect would be attenuation of ischemia, and consequently better perfusion of tissue in the post-traumatic state.

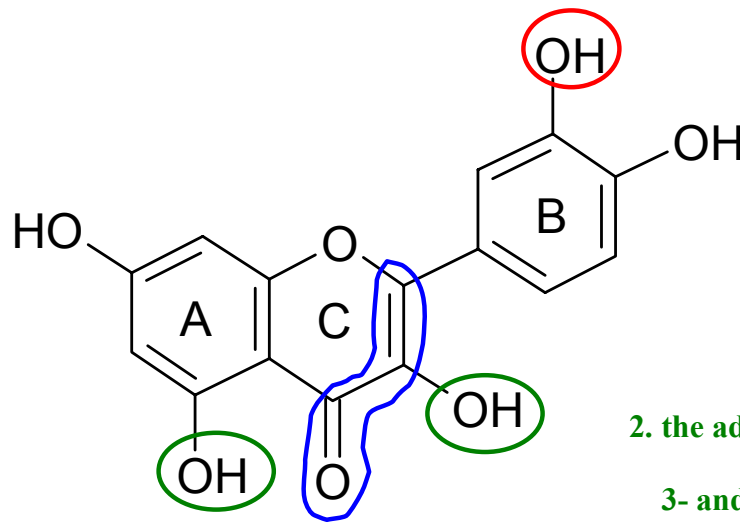
Anti-inflammatory and anti-edematous activity: In a model of chronic inflammation, quercetin significantly reduced cotton pellet-induced granuloma formation ($p < 0.05$), ranking in the best third of thirty tested flavonoids (Pelzer et al., 1998). In a rodent study of

carageenin-induced paw edema, a model of acute inflammation, quercetin has been found to significantly ($p < 0.05$) reduce the extent of edema (Pelzer et al., 1998). The anti-inflammatory activity of quercetin ranked second in a group of thirty flavonoids tested. The anti-inflammatory action of quercetin might be at least partially explained by the fact that the compound changes the secretion profile of mast cells and neutrophils (Bennett, 1981).

Antioxidative action: According to Bors (1990), three structural components are contributing to the anti-oxidant potential of the flavonoids (Fig. 4.6).

1. the 0-dihydroxyl (OH) structure (catechol)

in the B-ring



QUERCETIN

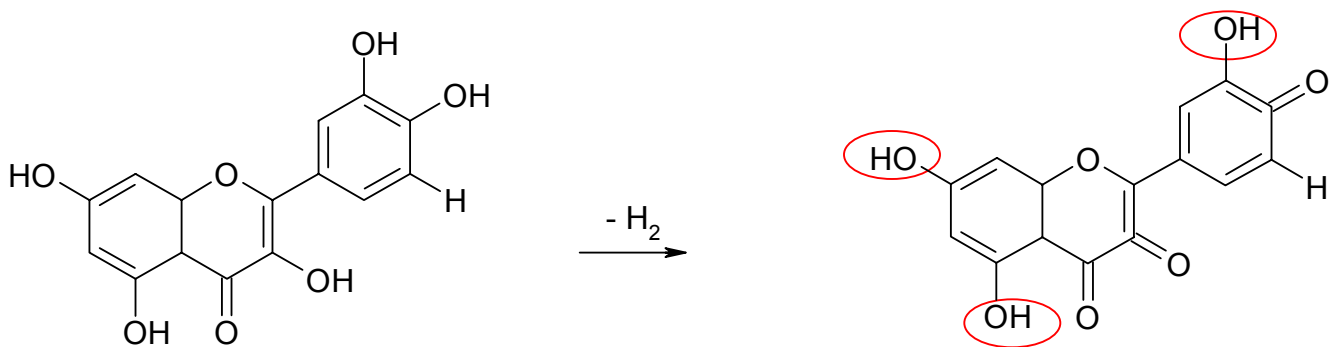
3. the 2,3-double bond in conjugation with a 4-oxo function

FIGURE 4.6: Structural components, on which the antioxidant capacity of quercetin is based.

Quercetin fulfills all those structural requirements. In fact, Wang and Joseph (1999) were able to show that, due to the presence of all three components required for anti-oxidative potential, quercetin was the flavonoid with the highest protective effect against H₂O₂ induced calcium dysregulation.

Iron chelation: Quercetin was found to have a high capacity to chelate iron (Kostyuk, 1998). When investigated for its ability to suppress the Fenton reaction, indicated by absorption spectroscopic parameters of the iron-ATP complex, it was shown that the reaction was suppressed completely with a minimum ratio quercetin to iron of 1.5 : 1 (Cheng and Breen, 2000). Thus, the chelation potential of quercetin ranked highest amongst the four flavonoids tested in this study. Quercetin was also shown to reduce Fe²⁺-induced peroxidation of unsaturated fatty acids (Vasilyeva, 2000). Using electrospray mass spectrometry, Fernandez and colleagues were able to provide direct evidence of chelate formation between iron ions and quercetin (Fernandez et al., 2002). In the same study, it was shown that quercetin, like all other flavonoids tested, was able to reduce Fe³⁺ to Fe²⁺, and the reaction stoichiometry between Fe²⁺ and quercetin was obtained as 1:1 or 1:2 (apparently both are possible). The reducing ability of quercetin ranked second place behind myricetin and before catechin. It was observed that this ranking corresponded with the redox potentials of the flavonoids tested. It has been suggested that the reducing ability might be based on the number of hydroxyl (OH) groups present in the molecules (Sichel et al. 1991; Cao et al., 1997; Fernandez et al., 2002). The redox potentials of flavonoids actually increase proportionally to the number of hydroxyl groups present in the molecules. The lower the number of the OH groups, the less is the probability that a hydrogen atom is freed to reduce

iron ions, a process that at the same time oxidizes the flavonoid. Even the oxidation products of quercetin still contain three or four hydroxyl groups (Fig. 4.7), therefore they should still be able to act as antioxidants (Jørgensen et al., 1998; Manach et al, 1998). However, since the pK_a of those oxidation products are considerably lower than quercetin, their redox potential is expected to be lower than that of quercetin itself (Laroff et al, 1972; Jovanovic et al., 2000).



QUERCETIN

FIGURE 4.7: Oxidized structure proposed for quercetin.

Even the oxidation products of quercetin still contain three or four hydroxyl groups, therefore metabolic products of quercetin should be active as antioxidants themselves.

from: Fernandez et al., 2002

Besides the hydroxyl groups, apparently also the ortho-catechol groups contribute to the reduction capacity of quercetin (Fernandez et al., 2002) (Fig. 4.8).

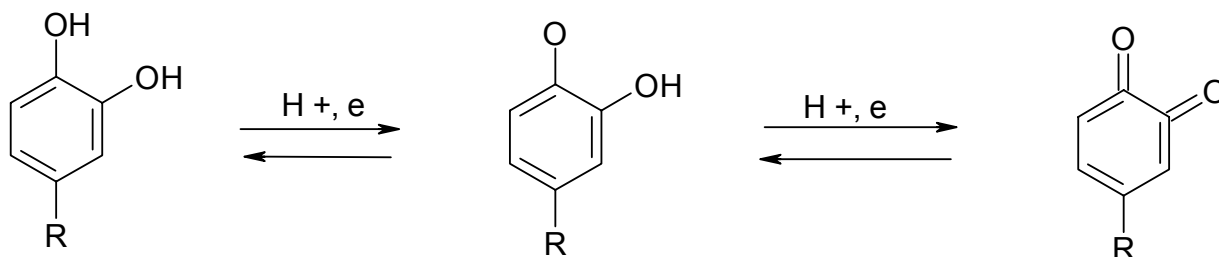


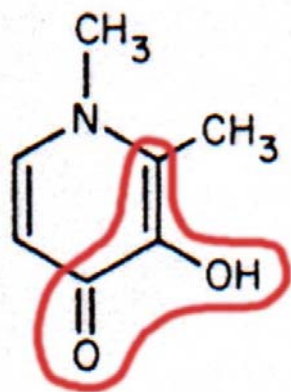
FIGURE 4.8: Oxidation of flavonoids possessing an oxo-catechol group.

from: Fernandez et al., 2002

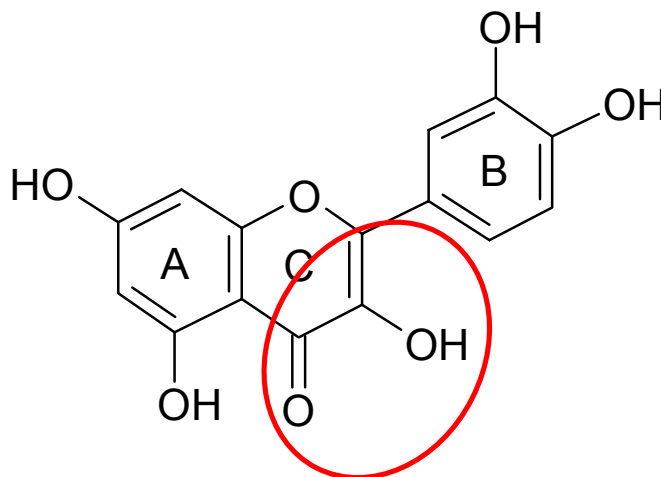
It is interesting to note that the redox potential of quercetin should be even higher than that of ascorbic acid (Laroff et al., 1972; Jovanovic et al., 1994).

Furthermore, possible sites of chelation in the quercetin molecule were identified in the same study. Morel and colleagues had proposed earlier three possible chelation sites for flavonoids containing the 4-oxo group and hydroxyl groups at 3, 5, 3' and 4' positions: between the 3-hydroxyl group and the 4-oxo group (Fig. 4.9), between the 5-hydroxyl and the 4-oxo group, and between the ortho-hydroxyl groups in the B-ring (Morel et al., 1998). From the results of their experiments, Fernandez and colleagues argue that the preferred site

for iron chelation possibly involves the 4-oxo group and 5-hydroxyl group (Fernandez et al., 2002, Mira et al., 2002).



DEFERIPRONE



QUERCETIN

FIGURE 4.9: Structural similarities for one of the possible iron binding sites between quercetin and deferiprone, a commercially available iron chelator active after oral administration.

This overview, illustrating that quercetin attenuates several pathological key mechanisms of CNS injury, supports the idea that the compound should be well suited for therapeutic intervention after CNS injury. My experiments, therefore, were designed to test the hypothesis that administration of quercetin attenuates secondary damage after CNS trauma. Studies were focused on the ability of quercetin to chelate iron, decrease oxidative stress (reflected by GSH levels), inflammatory processes (reflected by myeloperoxidase activity)

and trauma-induced apoptotic cell death in the setting of spinal cord injury and head trauma in the adult rat. The fluid percussion injury model for head trauma was used to test whether the actions of quercetin resulted in improved ability of traumatized neuronal structures to retain normal functions, as reflected by the amplitude of compound action potentials. Using the spinal cord injury model, recovery of motor function in previously paralyzed hind limbs was used to assess whether the actions of quercetin determined by biochemical, histological and immunohistochemical methods actually translated into improvement of functional recovery.

Chapter 5

Materials and Methods

5.1 Animal Care

For all experiments, adult male Wistar rats were used. Animals were housed and cared for in a temperature-regulated animal facility (ambient temperature 22 - 24°C) and exposed to a 12-hours light/dark cycle in accordance with the guidelines of the Canadian Council on Animal Care.

In human patients with spinal cord injury, modern management has dramatically improved prognosis with regard to survival time and quality of life. Cornerstones of care for the human paraplegic patient are meticulous skin care to avoid development of defects as potential focus of infection, and regular emptying of the urinary bladder to avoid urinary tract infections. The same principles should be applied in the care for paraplegic animals. The skin is inspected three times daily in the first week after injury and twice daily thereafter. Antibiotic ointments or powder are applied to skin defects. In rare cases, surgical repair of major skin defects was necessary. It is noteworthy that, where the majority of skin defects seen in human patients are pressure sores due to immobility and lack of proper sensation, this is rarely the case in our animal model, since even completely paraplegic animals exhibit a remarkable mobility within their cages. Rather, animals tend to suffer from self-inflicted injury to abdominal skin or extremities. This might be due either to lack of perceiving the body part devoid of sensation as “self” or from unintentionally aggressive grooming of those body parts. The urinary bladders are emptied three times daily for the first week after injury, until spontaneous bladder function resumes. This corresponds well to the advice for the care of human patients, where regular intermittent catheterization is preferred over indwelling catheters to avoid urinary tract infections. Adequate hydration, as advised for human patients, is realized by the animals’ ad libitum access to drinking water.

5.2 MRI for the study of iron chelation by quercetin

Contusional hemorrhage is seen in many cases after acute traumatic spinal cord injury, at either macroscopic or microscopic level. The lack of oxygen in the erythrocytes present in hemorrhagic tissue creates a situation of oxidative stress, which quickly depletes the glutathione content of the cells. Consequently, hemoglobin disintegrates and iron is released in chelatable form (Comporti, 2002). Disintegration products of hemoglobin further contribute to oxidative stress and apoptosis in the CNS (Matz, 2000; Gaetani, 1998). Chelatable iron products catalyze lipid membrane peroxidation in the central central nervous system, causing functional and structural loss of cells (Juurlink, 1998). Under the assumption that iron chelation can reduce oxidative damage after CNS injury, we tried to determine the dose at which quercetin would chelate iron ions in an optimal way. The sizes of tested doses were in the range of molecular equivalents to therapeutically used iron chelators. Since our hypothesis was that iron chelation is an important pathway through which quercetin would act in a neuroprotective manner, we performed a set of experiments to find the dose at which iron chelation by quercetin would be optimal.

T1 relaxation of the water (proton) signal was used to determine the dose for optimal iron chelation by quercetin. In order to simulate the distribution of ferrous and ferric iron ions throughout the spinal cord, we decided to use a phantom or 'physical likeness', in form of 2% agar in 15 ml Falcon tubes (Figure 5.1). Although all chemicals were mixed with agar in the fluid phase, the solidification of agarose was required for technical purposes before the MRI study could commence. Using a fluid mixture for analysis would have resulted in the presence of air bubbles in the field of analysis, which would have caused distortion of the

measured signal. Mixed in the agar were solutions of either ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) or ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in concentrations from 0.001 mM to 10 mM and quercetin in concentrations from 2.5 to 250 μM .



FIGURE 5.1 Spinal cord phantoms (agar in 15 ml Falcon tubes) for MRI analysis of iron chelation by quercetin dihydrate. Mixed in the fluid agar were solutions of either ferrous sulfate or ferric chloride with quercetin suspension in varying concentrations. Once the agar had solidified, the phantoms were submitted to MRI analysis.

Data were acquired on 3 mm slices with a 3 Tesla magnet (Magnex and Surrey Medical Imaging System, U.K.), operating at 130 MHz for protons. The imaging conditions for the saturation recovery relaxation experiments were FOV 50cm, TE 15 ms, matrix size 128 x 128. The resolution was 400 Hz in the frequency dimension and 0.3 mm in the spatial dimension. Relaxation times were varied from 100 ms to 3 s. T1 relaxation rates were computed by non-linear least squares fit to the equation

$$S_n = S_0(1 - e^{-TR_n/T1})$$

where S_n is the signal intensity in a given image pixel at relaxation time TR_n . Mean values were computed from individual pixel values in a circular region of interest near the center of a phantom using Cheshire™ image analysis software. Mean values were computed from individual pixel values in a circular region of interest near the center of a phantom using Cheshire™ image analysis software. Acquisition and interpretation of MRI data were performed by Dr. Ed Kendall and Zhao Ghong, Department of Medical Imaging, University of Saskatchewan.

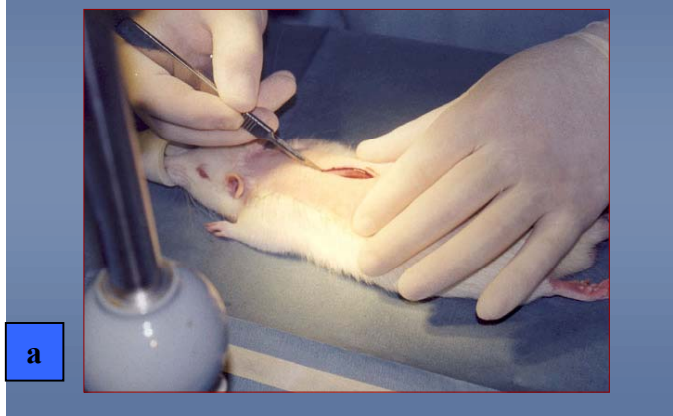
The major advantage of these preliminary MRI studies was that, for our animal studies, the number of groups receiving different doses of quercetin in the animal experiment was greatly reduced, since we were able to narrow down the potential dose range at which quercetin could be expected to be thearapeutically successful. This, of course, was provided that iron chelation would prove indeed an important pathway through which quercetin would act neuroprotective.

5.3 Surgical approaches

5.3.1 Spinal cord trauma

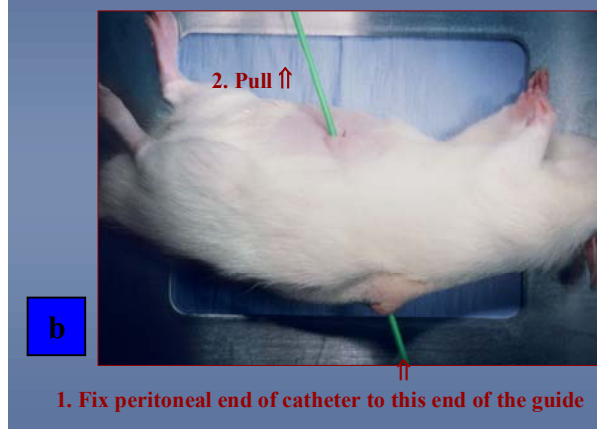
For all experiments, we used the model of spinal cord compression injury developed by Rivlin and Tator (Rivlin and Tator, 1978). Adult male Wistar rats (9-10 weeks of age, 285 – 360 g, Charles River Canada) were subjected to standardized mid-thoracic spinal cord injury at T7 level. We used 5% Halothane® in oxygen with a flow rate of 1.5 liter / min for induction and 2% Halothane® with the same oxygen flowrate for continuation of anesthesia. The back and the lower abdomen of the animals were shaved and disinfected with Hibitane® (chlorhexidine) and 70% alcohol. For analgesia, animals were pre-medicated with a subcutaneous injection of 0.05 mg Buprenorphine® / kg and received tapered doses in 12-hr intervals for 3 days. A vertical midline incision of the skin was performed over the spinous processes T5 to T7, and a catheter (Butterfly®-23 with cut-off needle) was tunneled subcutaneously towards the abdomen and implanted intraperitoneally for later repeated drug or vehicle application in all those animals who received drug or vehicle for longer than 24 hr (Fig. 5.2) It is our experience that these catheters significantly ease the repeated administration of intraperitoneal compounds.

Implantation of intra-peritoneal catheter



a) dorsal incision

Introducing a guide and the catheter



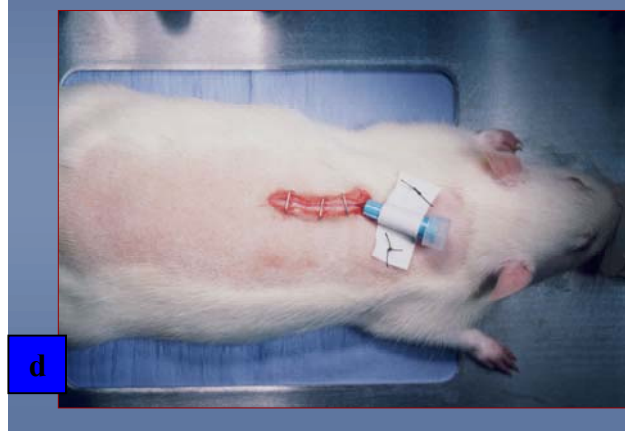
b) subcutaneous tunneling
(dorsal to ventral)

Butterfly 23 G, inserted sub-cutaneously (needle cut off before pulling through)



c) insertion of catheter (ventral to dorsal)

Fix catheter with tape and stitch to skin



d) catheter hub fixed to skin

FIGURE 5.2: Implantation of dorso-peritoneal catheter for frequent drug administration.

Catheters significantly ease repeated administration of intraperitoneal compounds.

The animal then was turned back into prone position, the dorsal incision was extended and muscles separated from T6 and T7 spines and laminae. Laminectomy was performed at T6/7 level and the spinal cord was exposed without opening the dura mater. An aneurysm clip (Kerr-Lougheed clip, Walsh Manufacturing, Oakville, Ontario; Fig. 5.3) with a calibrated closing force of 50 g (40 g for the experiments described in chapter 6) was closed around the spinal cord for 5 seconds (Fig. 5.4). The muscles were reapproximated with absorbable suture and the skin was stapled. The injection adapter of the catheter was secured with tape and sutured to the skin. Three additional animals received laminectomy only, but no spinal cord injury, to exclude impairment of neurological function by surgical trauma unrelated to the spinal cord injury.

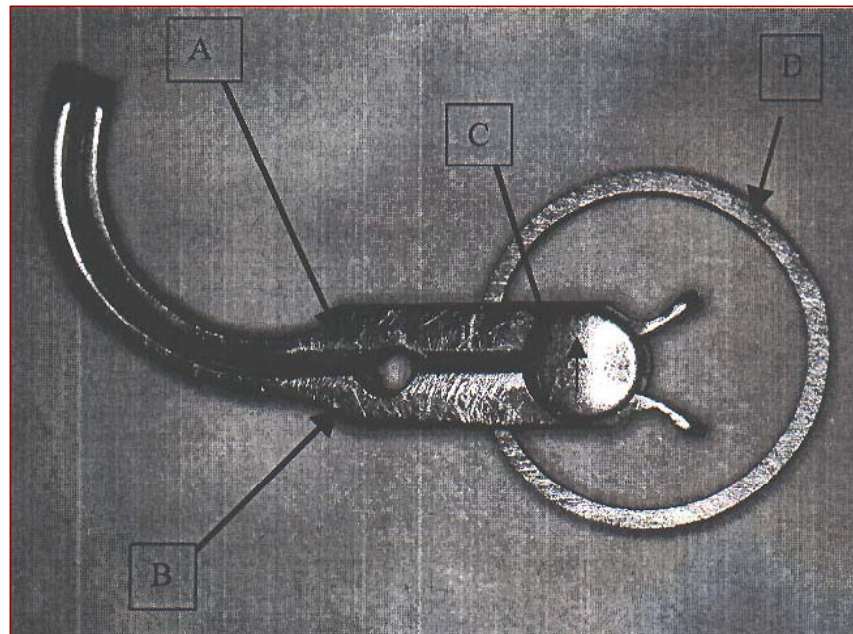
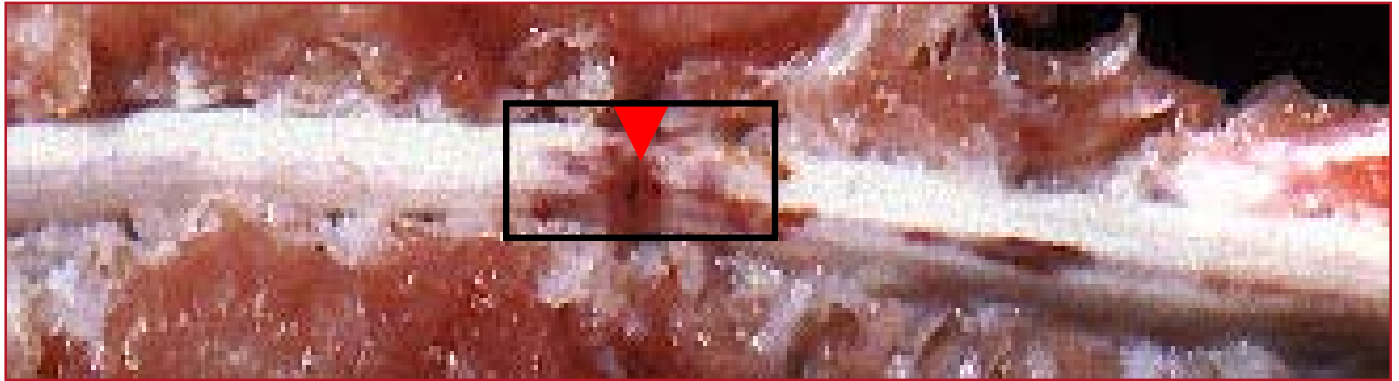
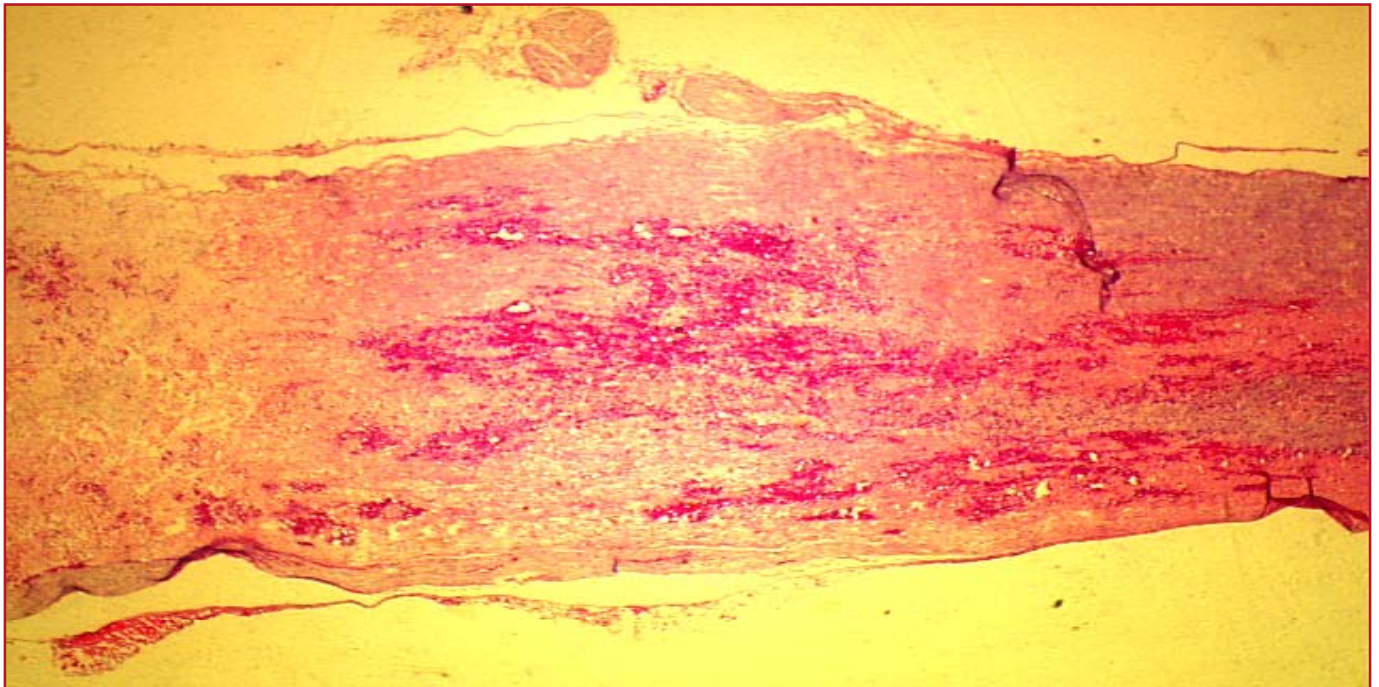


FIGURE 5.3: Kerr-Lougheed aneurysm clip. Closing force between the blades can be calibrated by varying strength of spring.

A: Upper blade B: Lower blade C: Roller D: Spring



- a) spinal cord in situ, dorsal portion of vertebral arches removed
Arrow: site of clip application visible within hemorrhagic zone



- b) longitudinal section of spinal cord, site of injury and adjacent spinal cord segments, corresponding to black rectangle in a)

FIGURE 5.4: Spinal cord of adult male Wistar rat, 12 hr after 50 g clip application for 5 sec.

Animals were weighed every other day and received either weight-adjusted doses of quercetin (quercetin dihydrate, Sigma) or saline vehicle only. Quercetin is poorly soluble in aqueous solutions and was suspended in physiological saline solution. The suspension was administered intraperitoneally via the implanted catheter or as direct i.p. injection for animals which received drug or vehicle for a maximum period of 24 hr. The urinary bladders of all paraplegic animals were expressed manually three times daily until recovery of spontaneous voiding was achieved. The latter was observed in most animals, whether treated with quercetin or not, between weeks 2 and 3 after injury.

With regard to survival time after injury, two basic types of experiments were performed: short-term experiments, with survival times between six hours and three days after injury, and long-term experiments, with survival times up to twelve weeks after injury.

5.2.1.1 Short-term experiments

Experiments in this group were designed to yield spinal cord tissue in which changes during the acute phase of spinal cord injury could be assessed. Animals were sacrificed at 6, 12, 24 or 72 hr after injury. Experiments in this complex were designed to prove that administration of quercetin attenuates inflammation and apoptosis in the early phase after acute traumatic spinal cord injury. Myeloperoxidase activity levels were measured in spinal cord tissue and serum of healthy, uninjured animals, quercetin-treated animals and saline controls at 6, 12, 24 and 72 hr after injury. Immunostaining for activated caspase-3 was performed on sections from animals sacrificed at 12, 24 and 72 hr after injury.

5.2.1.2 Long-term experiments of chronic injury phase

The assessment in these experiments centered around functional recovery after spinal cord trauma. While the purpose of the first phase of experiments was the determination of an effective dose, in later experiments we tried to establish the therapeutic window for quercetin administration.

5.2.1.2.1 Finding the optimal dose

In this early phase of experiments, we hypothesized that the capacity to chelate free iron ions was of foremost importance in the neuroprotective action of quercetin. The survival time after injury in this experimental block was 4 weeks, after which all animals were sacrificed by pericardiac perfusion with FAM (Formalin : Acetic acid : Methanol = 1 : 1 : 8).

In the first experimental block, animals were treated with different doses (0, 5, 25, 50 and 100 $\mu\text{mol/kg}$) of quercetin or saline vehicle. Treatment started 1 hr following injury and continued in 12 hr-intervals. Overall eight weight-adjusted doses of quercetin were administered to each animal in the therapeutic groups over a period of 4 days via the intraperitoneal catheter.

Based on the results from this first block of animal experiments and our preceding MRI experiments, we chose the dose of 25 $\mu\text{mol/kg}$ as optimal dose for further experiments. In the second block of animals, we extended treatment duration to 10 days. This was based on the rationale that significant amounts of free iron from hemoglobin disintegration would be present only beyond day two after injury, and they would persist for several days to follow. Extension of the treatment duration, therefore, would potentially increase the therapeutic benefit.

5.3.1.2.1 Defining the therapeutic window

A total of 104 adult male Wistar rats were subjected to standardized mid-thoracic spinal cord injury and assigned to nine therapeutic groups or used as saline controls. Age-matched, uninjured animals were used as healthy controls. Usually, surgery for two or three different experimental protocols was performed in parallel, and to these two or three experimental groups, animals were assigned randomly. Also, for all groups containing more than seven animals, surgery was performed on half of the animals at two different time points at least eight weeks apart, so as to verify the validity of results from the first part of the experiment.

In seven of the therapeutic groups, therapy started 1 hr after spinal cord compression injury. This time frame was chosen because it is the minimum time in which clinical assessment of a patient with spinal cord injury could be reasonably expected. In one group (group 7), treatment onset was delayed to 12 hr after injury. In one group (group 8), treatment onset was as late as 2 weeks after injury. The rationale for this fairly late treatment onset was to investigate whether by modulating pathological mechanisms occurring late after trauma, such as apoptosis and scar formation, a benefit for functional recovery was still achievable. Since the 25 $\mu\text{mol}/\text{kg}$ dose appeared to become less effective with prolonged interval between injury and treatment onset, we decided to use a higher dose of quercetin for the delayed treatment onset experiments. An additional group (group 9) was introduced to investigate whether the effects of early treatment onset and later stage high-dose therapy would potentiate each other.

Since our earlier studies had indicated that no statistically significant differences were seen between treatment duration of 4 and 10 days, several groups were introduced with shorter treatment duration, varying from one single injection to three days of treatment (groups 1 to 4).

Seven animals of Group 5, six animals of Group 8, all animals of Group 9 and six saline controls were allowed to recover for 12 weeks after injury, to assess whether the improvement of motor function would be permanent beyond the end of the treatment period. All remaining animals were sacrificed six weeks after injury.

Summary and description of the various experimental protocols used are provided in Table 5.1.

	Start treatment after SCI	Duration of treatment	Treatment schedule	Total dose administered
Group 1 n = 6	1 hr	single injection	25 µmol/kg	25 µmol/kg
Group 2 n = 5	1 hr	24 hr	25 µmol/kg twice daily	75 µmol/kg
Group 3 n = 12	1 hr	72 hr	25 µmol/kg twice daily	175 µmol/kg
Group 4 n = 6	1 hr	72 hr	25 µmol/kg three times daily	250 µmol/kg
Group 5 n = 14	1 hr	10 days	25 µmol/kg twice daily	500 µmol/kg
Group 6 n = 5	1 hr	10 days	25 µmol/kg three times daily	750 µmol/kg
Group 7 n = 12	12 hr	10 days	25 µmol/kg twice daily	475 µmol/kg
Group 8 n = 15	2 weeks	3 weeks	75 µmol/kg twice daily	3.51 mmol/kg
Group 9 n = 7	1 hr + 2 weeks	10 days + 3 weeks	25 µmol/kg twice daily + 75 µmol/kg twice daily	4.01 mmol/kg
Group 10 n = 23	1 hr, 12 hr or 2 weeks	24 hr – 3 weeks	3 ml saline / injection	no quercetin

TABLE 5.1: Summary of treatment protocols tested to establish the therapeutic window for quercetin administration.

n = animals in the group at beginning of the experiments

5.3.2 Moderate Head Trauma, modeled by Fluid Percussion Injury (FPI)

The clinical picture of head injury is much more varied than that of spinal cord injury. This is mainly due to the higher complexity of structure and function in the brain when compared to the spinal cord. Mechanisms of cellular pathology, however, are expected to be similar in both CNS compartments. Therefore, we investigated whether quercetin, if administered on a dosing schedule similar to that used in the spinal cord injury model, would also show beneficial effects in the setting of head trauma. This would answer a practical relevant question. Since spinal cord trauma is frequently associated with head trauma (Iida et al, 1999; Holly et al, 2002), it would be indeed good to test whether quercetin, when administered as therapy for spinal cord injury, would not be harmful or even beneficial in the setting of combined head and spine injury. Concussion in human head trauma can be closely modeled by fluid percussion injury to the brain, an animal model initially described by McIntosh and colleagues (1989). This model has been extensively characterized. It has the advantage that many of the features seen in human brain trauma are present in this animal model, such as axonal injury, neutrophil-mediated and non-neutrophil-mediated damage, neuronal loss and gliosis (Cortez et al., 1989; Hovda et al., 1995; Soares et al., 1995; Hill-Felberg et al., 1999; Graham et al., 2000). Furthermore, the force resulting in damage can be well controlled; the extent of brain damage is clearly related to force of injury (Perri et al., 1997)

We submitted adult male Sprague Dawley rats to moderate fluid percussion injury (2.0 to 2.2 atm) in anterior midline position, using the Virginia Commonwealth Impactor (Figure 5.5). The pressure of one atmosphere equals 14.7 pounds per square inch. Contrary to lateral fluid percussion injury, where the focus of tissue injury is found in the hippocampus

and damage detected mainly in the gray matter (Chen et al., 2003), with midline fluid percussion injury, the focus of damage is in the corpus callosum. Therefore, we were looking at a white matter injury. This appears to be particularly interesting in that the potentially salvagable structures in the setting of spinal cord injury are found in the white matter. In preparation of the procedure, animals in the experimental groups were anesthetized with a mixture of 2.5 % Halothane® in air. The heads of the animals were shaved and animals were placed on a warming pad to keep body temperature constant (controlled by rectal thermometer). Fixation in the stereotactic apparatus was performed with the fixation bars forming a straight trajectory through the openings of both external auditory canals. The shaven skin was disinfected with chlorhexidine and Betadine®, and sterile drapes were applied. After lowering the Halothane® content of the anesthetic mixture to 1.5 %, a 2 cm linear midline incision was performed to expose the periosteum. A burr hole of 4.5 mm diameter was set in the midline just behind the bregma. Using a fine rongeur, the burr hole then was extended to snugly fit the adapter for the fluid percussion transducer unit. Care was taken to avoid injury to the sinus and underlying dura mater. To achieve a complete seal between skull bone and the adapter, a mixture of cyanoacrylate (crazy glue) and acrylic powder was applied. Halothane® content in the anesthetic mixture was now reduced to 1 % and the seal was allowed to harden for 15 minutes. At this point, Halothane® was shut off completely, the connector tube from the fluid column was filled with warm normal saline and connected to the transducer unit. Once the animal started to regain consciousness, showing first spontaneous movements, the pre-set pendulum of the fluid percussion device was released to impact on the piston connected to the fluid column. Thus, a pulse of increased intracranial pressure was produced by fluid displacement from the column into the skull

cavity, resulting in temporary compression and displacement of the brain tissue underlying the point of impact. A digital oscillograph recorded the pressure generated by the fluid column, verifying that indeed all animals had been submitted to moderate fluid percussion injury. All animals arrested breathing for an interval of variable length (4 seconds and longer), immediately following the fluid percussion injury.

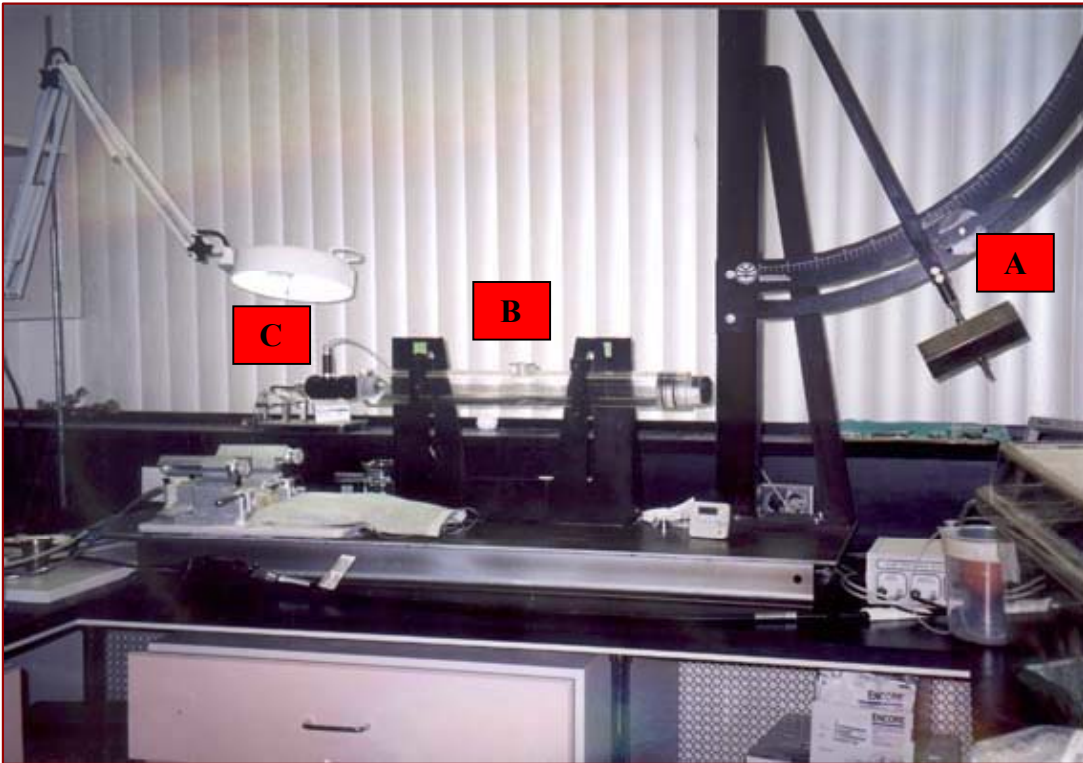


FIGURE 5.5: Experimental set-up for fluid percussion injury.

- A Pendulum with piston
- B Fluid column
- C Connection to transducer unit

Severity of the resulting injury is determined by the angle and height from which the metal pendulum is dropped onto the rubber piston that is connected to the fluid column.

If post-traumatic respiratory arrest was longer than 20 seconds, support was given with short-term artificial respiration. Four of the 26 animals required short-term artificial respiration after the injury. Immediately after the injury, inhalation anesthesia with Halothane® was re-induced, the seal between bone and adapter was carefully broken and the adapter removed. Dura and sinus were checked for intactness and the adapter was checked for patency. The burr hole was plugged with bone wax, skin was stapled and the fixation bars of the stereotactic apparatus were removed. Inhalation anesthesia was terminated and animals were placed on a warming pad in the recovery cage.

Injured animals were divided into two groups: one group received 25 µmol/kg quercetin intra-peritoneally, starting 1 hour after injury with continued treatment in 12-hour intervals, while animals in the second group received weight-adjusted doses of normal saline according to the same schedule (n = 13 per group). Out of each group, five animals were sacrificed 24 hr after injury, and five animals were sacrificed 72 hr after injury by Halothane® overdose and decapitation. All animals received their last injections about one hour before sacrifice. While animals used for electrophysiology experiments were beheaded and brains immediately extracted, animals used for histological and biochemical analysis were sacrificed by pericardiac perfusion with ice cold saline.

Electrophysiology: The separated heads were placed in ice cold (< 3° C) artificial CSF (ACSF) saturated with 95% oxygen and 5% CO₂ at pH 7.4 and brains were extracted within 2 minutes of death. The composition of the ACSF was 126 mM NaCl, 3 mM KCl, 1.4 mM KH₂PO₄, 2.4 mM CaCl₂, 1.3 mM MgSO₄, 26 mM NaCO₃ and 10 mM glucose. Brains were mounted on a vibrotome and 400 µm coronary sections were cut from the potential

injury area, which included the corpus callosum. Compound Action Potentials (CAP) were recorded on those vibrotome sections, while superfusion was continued with artificial CSF at body temperature, saturated with 95% O₂ and 5% CO₂ and delivered at a flow rate of 6-8ml/min. For recording, slices were submerged in a closed, box-like superfusion chamber with small slits which allowed access to the electrodes (Tian and Baker, 2000). The CAPs were evoked by electric stimulation and recorded extracellularly at 1.0 mm from the stimulation site in the corpus callosum. The typical evoked CAP includes 3 negative peaks, and the CAP amplitude was quantified as voltage difference of the peak-to-peak measurement between the second negative peak and the positive peak preceding it. CAPs of injured animals were compared with CAPs of 5 healthy control animals. Acquisition and interpretation of CAPs were performed by Ming Zhao and Guo-Fen Tian from the Cara Phelan Trauma Centre at St. Michael's Hospital in Toronto.

Measurements of action potentials after brain trauma for assessment of posttraumatic damage and recovery have been used in several laboratories (Akasu et al., 2002; Ross and Soltesz, 2000; Reeves et al., 1995). To our knowledge, no published data exist where CAPs have been used to assess protection / recovery after therapeutic intervention.

5.4 Assessing recovery of motor function

As Langworthy (1970) points out in his review of the studies of Derek Denny-Brown, movement is a series of postures, with each movement itself being a modification of the preceding posture. After injury to the nervous system, reflex motor responses to sensory stimuli might be lost. The resulting defect might be a loss of motor function and the ability to maintain posture, depending on location and extent of the trauma. In conclusion, a valid method for assessment of therapeutic efficacy would have to include standardized and easily reproducible measurements regarding differences in posture, with which the extent of recovery of motor function in the initially paralyzed extremities of experimental subjects can be assessed.

Behavioral testing for all long-term animals was performed once weekly. For assessment of motor function recovery in the hindlimbs, the BBB scale and inclined plane test (Angle board scores) were used.

5.4.1 Angle board score

Eidelberg and colleagues used an inclined ramp to assess motor function in spinal cord-injured ferrets (Eidelberg et al., 1976). The system was consequently adapted for rats by Rivlin and Tator (1977). Other than with the original Eidelberg system, no operant conditioning, i. e. no training of the animals previous to the test situation, is required with this modified scoring system. Rats are placed horizontally on an inclined plane, and the score assigned is the maximum angle at which the rat can maintain its position for a minimum of five seconds without falling. According to Rivlin and Tator, normal, i.e. non-injured rats were able to hold position up to about 80°, while rats with transected cords maintained

themselves at angles around 23°. The angle at which uninjured, healthy rats can maintain position on the inclined plane depends very much on the surface material used. In our experience, healthy, uninjured rats maintained themselves at levels between 45 - 55° (Fig. 5.6). Differences in scores obtained for healthy rats in other laboratories are most likely due to differences in the surface of the Angle boards used. While the scores of this test system give us some information about the strength in the hind limbs, we do not gain significant information about posture and voluntary limb positioning.



FIGURE 5.6: Angle board test.

The non-injured rat is able to hold position on the inclined plane raised to an angle of 50°.

5.4.2 BBB score, developed by **B**asso, **B**eattie and **B**resnahan

The BBB scoring system, on the other hand, assesses motor function through posture and positioning of the hind limbs in an open field test, thereby relaying indirectly also information about strength in the hind limb muscles (Basso et al., 1995). Animals were acclimatized to the test situation twice daily for 5 minutes, for at least 4 days before surgery (Fig. 5.7). With the BBB scoring system, points on a scale from 0 to 21 are awarded to assess posture and positioning of the hind limbs. Zero denotes no noticeable movement in the hind limbs, while 21 describes a perfectly healthy, walking animal (Fig. 5.8). It has been shown in a multi-center study that the inter-observer difference amounts to two points (Basso et al., 1996). However, from a clinical aspect, a difference of one point on the BBB scale could be significant, where it denotes the difference between walking and non-walking animals. Assessment was performed by two observers, of whom at least one was blinded to the treatment.



FIGURE 5.7: Animals for long-term experiments are acclimatized to handling and to the test situation twice daily for 5 minutes, for at least 4 days before surgery. Although shy on arrival, they soon adapt to the new environment and start exploring the new space.

Score	Verbal description of hind limb movement
0	No observable hind limb movement
1	Slight movement of one or two joints
2	Extensive movement of one joint <i>or</i> extensive movement of one and slight movement of another joint
3	Extensive movement of two joints
4	Slight movements of all three joints of the hind limbs
5	Slight movements of two joints and extensive movement of the third joint
6	Extensive movements of two joints and slight movement of the third joint
7	Extensive movement of all three joints
8	Sweeping without weight support <i>or</i> plantar paw placement without weight support
9	Plantar paw placement with weight support in stance only
10	Occasional weight supported plantar steps, no fore limb (FL) – hind limb (HL) coordination
11	Frequent to consistent weight supported plantar steps and no FL - HL coordination
12	Frequent to consistent weight supported plantar steps and occasional FL – HL coordination
13	Frequent to consistent weight supported plantar steps and frequent FL – HL coordination
14	Consistent weight supported steps, consistent FL – HL coordination, paw rotated on initial contact
15	Consistent plantar stepping and FL – HL coordination; no toe clearance during forward limb advancement; paw position predominantly parallel to body at initial contact
16	Consistent plantar stepping and FL – HL coordination; toe clearance frequently during forward limb advancement; paw position predominantly parallel to body at contact and rotated at lift off
17	Consistent plantar stepping and FL – HL coordination; toe clearance frequently during forward limb advancement; paw position predominantly parallel to body at contact and lift off
18	Consistent plantar stepping and FL – HL coordination; toe clearance consistently during forward limb advancement; paw position predominantly parallel to body at contact and rotated at lift off
19	Consistent plantar stepping and FL – HL coordination; toe clearance consistently during forward limb advancement; paw position predominantly parallel to body at contact and lift off
20	Consistent plantar stepping and FL – HL coordination; consistent toe clearance; paw position predominantly parallel to body at contact and lift off; tail consistently up; trunk instability
21	Healthy animal (consistent stepping, FL-HL coordination, proper paw position, tail up, trunk stability)

Table 5.2 BBB score assessment (after Basso et al, 1995)



FIGURE 5.8: Weekly BBB scoring as assessment of spontaneous hind limb movement.



a): BBB 1 = slight movement in one or two joints.



b): BBB 7 = extensive movement of all three joints.



c): BBB 14 = consistent fore limb – hind limb coordination, weight supported steps.

5.5. Histology

At the end of the observation period, animals were sacrificed under Halothane® anesthesia by intracardiac perfusion. In the first step of perfusion, we used 1% sodium nitrite in PBS (1 ml/g rat). Further processing differed between the experiments. For the experiments described in chapter 6, the first step was followed by perfusion with similar volumes of FAM (Formaldehyde : Acetic acid : Methanol = 1 : 1 : 8). Spinal cords were isolated and embedded in paraffin two days later. For all other experiments, perfusion with 4% paraformaldehyde followed the first step. Spinal cords were isolated and stored in 4% paraformaldehyde overnight, after which they were switched to 30% glucose (as cryoprotectant) for 48 hr. For analysis of tissue from rats submitted to moderate head trauma, frozen coronary sections of 20 µm thickness were mounted on gelatin-coated glass slides. Alternating sections were stained with Hematoxylin & Eosin (H&E stain) and LFB-Cresylviolet stain.

Hematoxylin & Eosin (H&E): This inexpensive, easy to perform stain allows good histological orientation about tissue structures, including the formation of post-traumatic cavities within the spinal cord. Hematoxylin is a basic dye and intercalates with acidic tissue structures, staining them in shades of blue (i.e. cellular nuclei). Eosin, an acidic dye, stains cytoplasmic structures in shades of pink.

Luxol Fast Blue (LFB)-Cresylviolet: Luxol Fast Blue stain is used to distinguish myelin sheaths, which appear blue, in contrast to structures containing Nissl substance, which stain pink-red.

Mallory stain for iron: This stain is used to demonstrate presence or absence of ferric iron (Fe^{3+}) in the tissue (Mallory, 1961). Animals were sacrificed by pericardiac perfusion with 1% nitrite in PBS and FAM at 4 weeks after spinal cord injury. Sections of 10 μm thickness from the center of the injury and adjacent segments were mounted, deparaffinized in a xylene-to-alcohol series and immersed in a 5% hydrochloric acid – potassium ferrocyanide solution (1 : 1) for 20 minutes. Ferric iron stains light blue. After rinsing with distilled water, sections were counterstained with nuclear fast red in 5% aluminum sulfate for 5 minutes.

Caspase-3 expression: Animals were sacrificed by perfusion under inhalation anesthesia with Halothane®, using 1% sodium nitrite in PBS (1 ml/r rat) and equal volumes of 4% paraformaldehyde (PFA). Spinal cords were isolated immediately and stored in 4% PFA overnight, after which they were transferred into 30% sucrose solution for 48 hr. Spinal cords were then cut to pieces containing three spinal cord segments and embedded in OTC. Cryosections of 20 μm thickness were mounted on Superfrost plus® slides (VWR) and stored at -70°C until further processing. An anti-human / mouse caspase-3 antibody (R&D System Inc.) that recognizes only activated caspase-3 was used in a dilution of 1:500 to assess whether administration of quercetin inhibited caspase-3 activation in vivo. Slides with frozen sections were equilibrated to room temperature for 1 hr and washed with PBS. The slides were immersed in methanol and H_2O_2 (1:3) for 30 minutes, followed by three 5 minute

wash cycles with PBS. Blocking serum was applied for 2 hr, after which sections were incubated with the primary antibody overnight. After another three 5 minute wash cycles with PBS, the secondary antibody (polyclonal anti-rabbit, Vector) was applied for 1 hr. The sections were washed again with PBS, and the slides were finally developed using DAB kit (Vector) according to instructions. Semi-quantitative assessment of staining was performed at light microscopic level.

5.8 Biochemistry

Spectrophotometric assay for myeloperoxidase: Animals were sacrificed by pericardiac perfusion under inhalation anesthesia with Halothane®, using about 300 ml ice cold physiological saline per animal. Before saline was introduced, about 3 ml of blood were aspirated. The whole blood was then centrifuged for 3 minutes at maximum speed. The fluid phase was aspirated and stored in cryovials at -70°C until biochemical assays were performed. The spine-muscle blocks were isolated quickly and cooled by pouring liquid nitrogen over the tissue repeatedly. The isolated tissue blocks were fixed on a corkboard, and serial laminectomy was performed from mid-cervical to lumbar levels, while cooling with liquid nitrogen continued at regular intervals. Finally, the spinal cord tissue was harvested as single segments and stored in cryovials at -70° C awaiting further analysis. Myeloperoxidase activity was measured in both spinal cord tissue and plasma.

For the measurement of myeloperoxidase activity in spinal cord tissue, the spectrophotometric method as described by Carlson and colleagues (Carlson et al., 1998) was used. Briefly, spinal cord segments from the site of injury (T6-8) and segments cranial (T4) and caudal (T10) to the injury site were homogenized mechanically and sonicated in 50 mM HTAB (phosphate buffer, pH 6, containing 0.5% hexadecyltrimethylammonium bromide) on ice, two times for 3 seconds and once for 5 seconds. Hexadecyltrimethylammonium bromide is a detergent used to extract MPO from the neutrophil granules (Krawisz et al, 1984). The sonicated homogenates were centrifuged for 15 minutes at 13,000 rpm and 4° C, after which the supernatant was transferred to a new centrifuge tube. Absorbance at 460 nm was measured after adding o-dianisidine dihydrochloride in potassium phosphate buffer (pH 6.0)

with H₂O₂ to the supernatant, using a spectrophotometer (SPECTRA MAX 190, Molecular Devices). Absorbance was calculated by computer software in the Endpoint protocol. Since the basis of this assay is the reaction of hydrogen peroxide with o-dianisidine dihydrochloride to form hypochlorous acid, one unit of myeloperoxidase activity is defined as the amount degrading 1 μmol of peroxide per minute at 25° C. For measurement of myeloperoxidase activity in serum, the serum collected at sacrifice of the animals was diluted 1:2 with HTAB. From there, the same procedure as described above for the spinal cord tissue was used. All analyses were performed in triplicate.

Glutathione assay: The tissue content of reduced glutathione content was determined in brains from rats submitted to moderate head trauma, using the monochlorbimane (mCB) fluorometric method as described in Kamencic et al. (2000). The homogenized frozen brain samples were weighed, thawed and sonicated in 20 vol of cold 50 mM Tris buffer (pH 7.4). Monochlorbimane was added to a final concentration of 100 μM along with 1 U/ml glutathione S-transferase obtained from equine liver, after which samples were incubated at room temperature for 30 minutes. The concentration of the GSH-mCB adduct was calculated from measurements using a Labsystems Fluoroskan II microtiterplate reader with excitation at 380 nm and emission at 470 nm. Each sample was assayed in triplicate. Protein content was determined using the BCA method as described by Smith and colleagues (Smith et al., 1985). GSH content was expressed as nmol GSH / mg protein. Determination of glutathione levels was performed by Dr. Huse Kamencic.

Chapter 6

QUERCETIN PROMOTES FUNCTIONAL RECOVERY FOLLOWING ACUTE SPINAL CORD INJURY

Adapted from:

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6.1 Introduction

The clinical picture of acute traumatic spinal cord injury is characterized by edema, inflammation and vascular changes. The latter include ischemia, impaired autoregulation, vasospasm and contusional hemorrhages (Tator, 1991; Tator and Fehlings, 1991). Hemorrhages can be found at microscopic level even in cases where, after spinal fracture, the spinal cord appears intact at macroscopic level (Schültke and Harruff, 2000). In cases of compression injury of the spinal cord, hemorrhages are usually visible at the macroscopic level. Under conditions of hemorrhage when red blood cells are removed from the high oxygen environment of the circulation, oxyhemoglobin forms deoxyhemoglobin; the deoxyhemoglobin undergoes denaturation to methemoglobin while the heme iron becomes oxidized from the ferrous Fe^{2+} to the ferric Fe^{3+} form (Bradley, 1993). Consequently, hemoglobin disintegrates and iron is released in chelatable form (Comporti et al., 2002). The disintegration products of hemoglobin contribute to oxidative stress and apoptosis in the CNS (Gaetani et al., 1998; Matz et al., 2000). The fact that ferritin is upregulated and transferrin is downregulated following spinal cord injury in humans (Koszyca et al., 2002) indicates that release of free iron ions occurs following spinal cord injury in humans.

Chelatable iron products catalyze lipid membrane peroxidation in the central nervous system, causing functional and structural loss of cells (Juurlink and Paterson, 1998). The iron ions released from hemoglobin accumulated in tissues can also catalyze the formation of hydroxyl radicals from hydrogen peroxide (Gutteridge et al., 1981) generated by phagocytes attracted to the site of injury. Hydroxyl radicals in turn contribute significantly to the production of lipid free radicals from polyunsaturated fatty acids and in the presence of molecular oxygen initiate chains of lipid peroxidation (Braugher and Hall, 1992). Fe^{2+} and

Fe^{3+} catalyze the formation of alkoxyl radicals and peroxy radicals respectively from the formed lipid hydroperoxides, thereby promoting the lipid peroxidation cascade (Halliwell and Gutteridge, 1989). Hence, any compound acting as an iron chelator ought to decrease damage created by secondary injury in the setting of CNS injury.

Some polyphenolic compounds such as quercetin efficiently chelate iron (Afanas'ev et al., 1988; van Acker et al., 1995; Morel et al., 1993; Romanova et al., 2001). Quercetin is a flavonoid. Flavonoids are diphenylpyrones having two benzene rings (A and B) linked by a heterocyclic pyrone ring. The critical molecular feature of many iron chelators is the presence of a bidentate structure as represented by carbonyl and an adjacent hydroxyl moiety or two adjacent hydroxyl moieties (Hider et al., 1994; Galey, 1997). Such a bidentate structure is present in the 3 (or 5) hydroxyl and 4 carbonyl as well as the 3' and 4' hydroxyl groups of quercetin (Fig. 1). Electrospray mass spectrometry studies indicate that it is the 5 hydroxyl and the 4 carbonyl moieties that are involved in quercetin's ability to chelate Fe^{2+} (Fernandez et al., 2002). Not only does quercetin chelate Fe^{2+} but it has been found to suppress the Fenton reaction (Cheng and Breen, 2000) and decrease Fe^{2+} -mediated peroxidation of unsaturated fatty acids (Ratty and Das, 1988; Vasilyeva et al., 2000). Furthermore, quercetin, contrary to its rutinoside rutin, is able to cross the cell membrane of erythrocytes (Sorata et al., 1984; Ben-Hur et al., 1993).

In the present study we provide proof-of-principle data indicating that quercetin as a therapeutic intervention following spinal cord injury should be pursued further.

6.2 Materials and Methods

MRI examination of iron chelation: There is a linear relationship between Fe^{2+} concentration and shortening of the T1 relaxation time (Vymazal et al., 1996). A T1 relaxation experiment was, therefore, performed to determine the relaxation rate of water protons in the presence of different concentrations (1-100 μM) of FeSO_4 . The factorial experiment was expanded by including increasing concentrations of quercetin (0-100 μM), an agent known to preferentially chelate Fe^{2+} ions. Data were collected on phantom spinal cords constructed in 15 ml culture tubes containing 2% agarose in physiological saline as described in section 5.2.

First animal experiment: Twenty-eight animals underwent standardized spinal cord compression injury as described in section 5.3.1. The animals were randomly assigned to 4 therapeutic groups and 1 saline control group ($n = 6$ in each) using a single blinded protocol. Animals were treated with different doses (0, 5, 25, 50 and 100 $\mu\text{mol/kg}$) of quercetin (Sigma, St. Louis, MO) or saline vehicle 1 hr following injury and then every 12 hr. Studies have shown that the serum half-life of intravenously administered flavonoids is about 12 hr (Griffiths and Barrow, 1992) and plasma half-life of orally administered quercetin of 4 hr with an elimination half-life of 16 hr (Hollman et al., 1996); hence, administration at 12 hr intervals appeared reasonable. Since the capacity of iron chelation was the targeted property in our compound, the quercetin dose range chosen based on MRI data. Quercetin dihydrate, poorly soluble in aqueous solutions, was suspended in physiological saline solution. The suspension was autoclaved for 20 minutes at 121 °C and consequently aliquoted into pre-sterilized vials. Rats were weighed daily, and an overall of 8 weight-adjusted doses of quercetin dihydrate were administered to each animal in the therapeutic groups over a period

of 4 days via the intraperitoneal catheter at 12 hr intervals. Three additional animals received laminectomy only, but no spinal cord injury, to exclude impairment of neurological function by surgical trauma unrelated to the spinal cord injury.

Behavioral testing, using BBB and Angle board scores as described in section 5.4, was performed once weekly for all animals by at least two observers blinded to the treatment. A crude assessment of nociception was also performed. This was done by pinching both hindlimbs and the tail with a straight, non-toothed forcep and then observing for withdrawal response.

Second animal experiment: This experiment was designed to answer two questions: 1) To determine the dose at which quercetin shows no therapeutic effect, and 2) Determine whether a treatment period longer than 4 days gave rise to an enhanced therapeutic effect. Sixteen animals were randomly, and blinded to the researcher, assigned to two therapeutic groups and one control group. Mid-thoracic spinal cord injury was produced following the same procedure as described above. Animals in the first therapeutic group (n=4) received 2.5 $\mu\text{mol/kg}$ quercetin and animals in the second therapeutic group (n=6) received 25 $\mu\text{mol/kg}$, whereas animals in the control group received physiological saline solution in weight-adjusted doses (n=6). Treatment was started 1 hr following injury and then every 12 hr for a period of 10 days.

Iron staining: Mallory stain for iron was performed on sections from the site of injury and adjacent spinal cord segments, as described in section 5.5.

6.3 Results

MRI examination of iron chelation: Neither quercetin in agarose nor the 2% agarose exerted an effect on T1 signal. Fe^{2+} ions originating from FeSO_4 (1-100 μM) in aqueous solution caused a shortening of the relaxation rate of the T1 (proton) signal. When quercetin was added to the aqueous FeSO_4 solution in various concentrations, the iron relaxation effect on the T1 signal was significantly counteracted (Fig. 6.1). Changes of the T1 relaxation rate caused by Fe^{2+} ions originating from FeSO_4 in aqueous solution were maximally antagonized by quercetin at a concentration of 25 μM . Further increases in quercetin concentration had no additional effect.

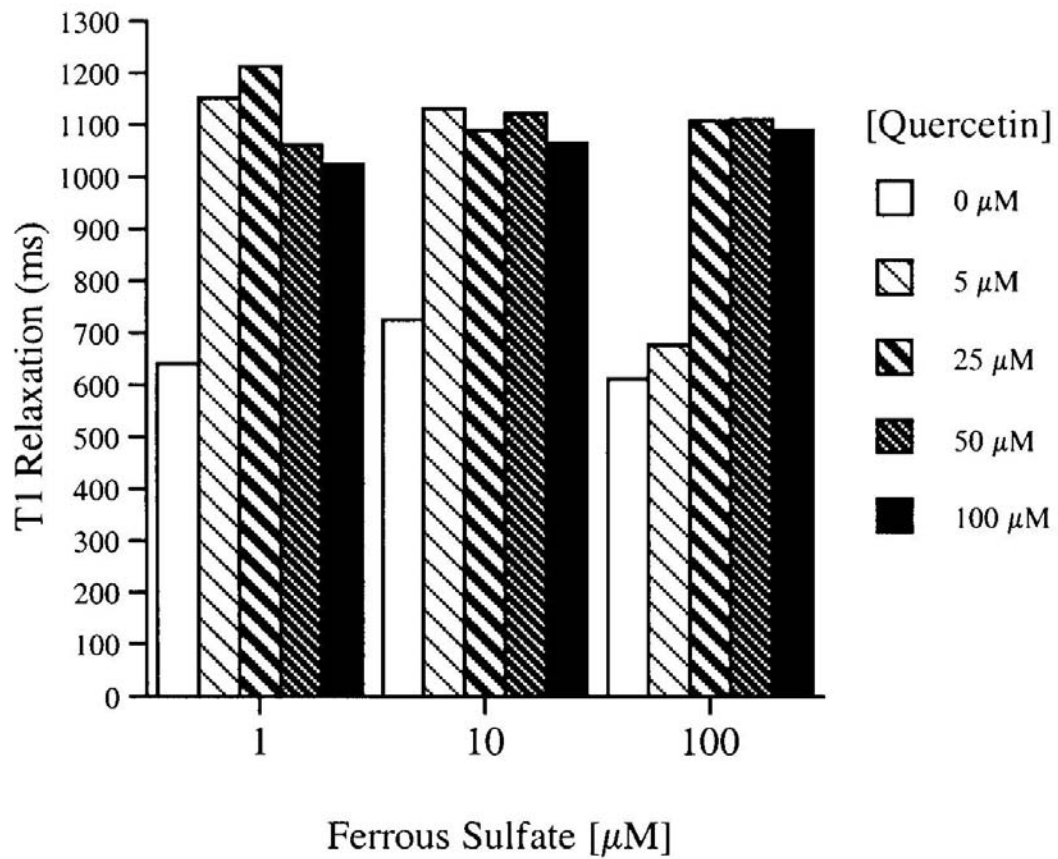


FIGURE 6.1: Impact of the presence of quercetin on iron-induced T1 signal relaxation.

The shortening of the T1 proton signal caused by Fe^{2+} ions in the agarose phantom is partially reversed by quercetin.

First animal experiment: All rats were completely paraplegic after the injury. Bladder function was lost and animals required manual expression of the bladder three times daily for about two weeks, after which bladder function was gradually recovered in most of the animals (in treated as well as in control animals).

At 1 week, nociception was recovered in both hind limbs in all but 1 animal (4.2%) while nociception was recovered in the tail in all but 5 animals (20.8%). There were no significant differences in distribution of findings throughout the various treatment groups. Nociception in both hind limbs and tail were recovered in all animals by 2 weeks after the injury.

The mean BBB scores for the different treatment groups are indicated in Fig. 6.2. Three of the 6 animals in each of the four quercetin treatment groups had BBB scores of 10 or greater, but not one of the saline treated animals achieved a BBB score greater than 9. The range in BBB scores for saline were (8, 8, 9, 8, 8, 6) and for quercetin-treated groups were 5 $\mu\text{mol/kg}$ (16, 8, 11, 14, 9, 7), 25 $\mu\text{mol/kg}$ (14, 8, 8, 16, 15, 8) 50 $\mu\text{mol/kg}$ (8, 12, 8, 11, 15, 7), 100 $\mu\text{mol/kg}$ (20, 8, 8, 16, 10, 7). There were no significant differences amongst the four quercetin-treatment groups but there was a significant difference between the quercetin-treated groups and saline vehicle-treated group ($P < 0.0012$, Welch's *t*-test).

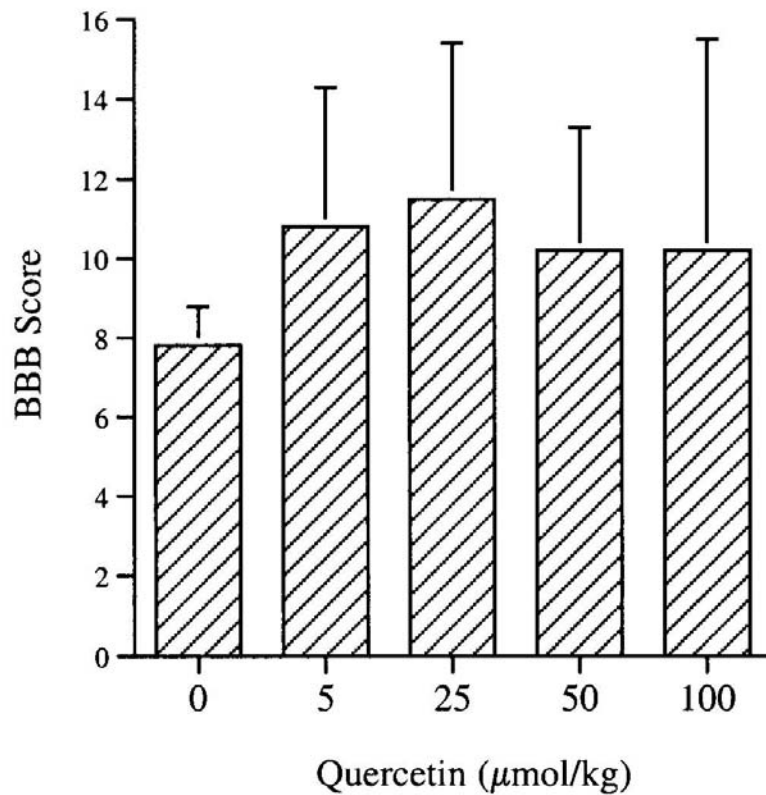


FIGURE 6.2: BBB scores at 4 weeks after mid-thoracic spinal cord injury with 40 g clip (first experiment). Treatment was with saline or quercetin at 5, 15, 50 and 100 μmol/kg. Depicted are means ± SDs. There are no statistically significant differences amongst the four treatment groups while there is a significant difference between quercetin-treated animals (all groups pooled) and the saline controls ($p < 0.0012$, Welch's test).

Second animal experiment: In this set of experiments we wished to test whether the length of treatment affected outcome and also to determine whether a dose of 2.5µmoles/kg would have a therapeutic effect. Animals were treated with quercetin at 2.5 or 25 µmoles/kg or saline vehicle for 10 days. BBB score outcomes for this experiment are illustrated in Fig. 6.3. With the lower dose group, only 1 out of 4 animals achieved a BBB score of 10 or better (BBB scores were 8, 8, 8, 16) by 4 weeks. Five out of 6 animals in the group treated with the higher dose of quercetin were weight bearing and walking at week 4 following injury. The quality of walking in this group, as rated according to BBB scale, varied between 11 and 20 (BBB 8, 11, 12, 14, 16, 20). In the control group receiving saline vehicle only, not one of the animals was walking at any time (BBB 3, 8, 8, 8, 8, 9). While improvement between week 1 and week 4 was not significant for the group treated with the lower drug dose, the performance in the group treated with the higher dose of quercetin improved significantly during the same time period. The difference in outcome between the therapeutic group receiving the lower dose and saline controls was not significant (ANOVA with post-hoc Tukey test). The animals treated with the higher quercetin dose did significantly better than the saline-treated controls ($p < 0.05$, ANOVA with post-hoc Tukey test).

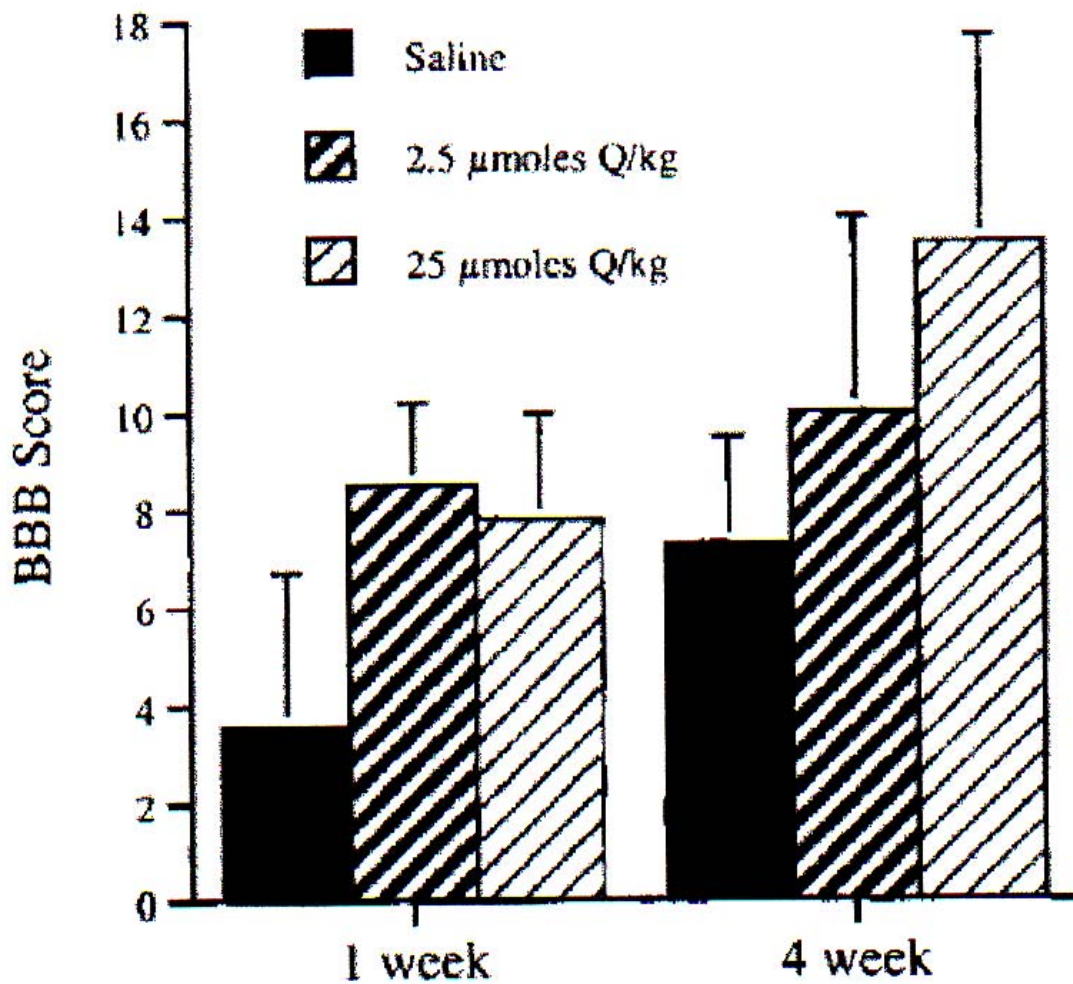
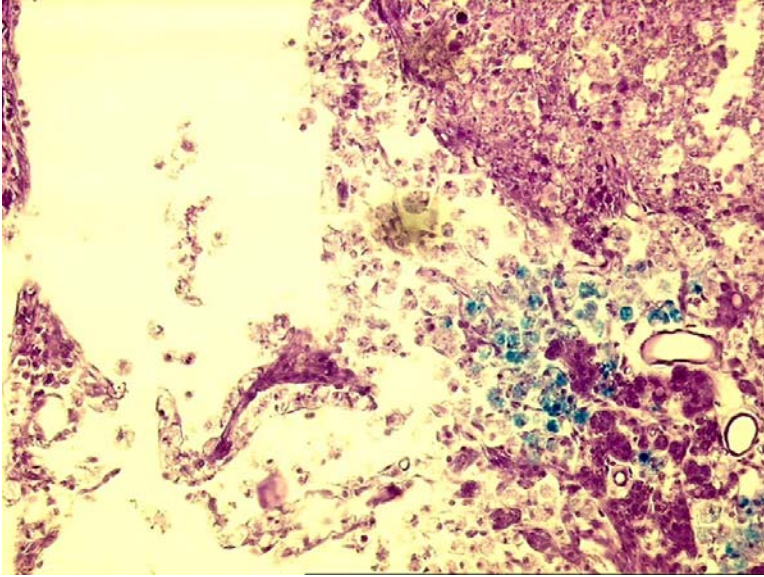
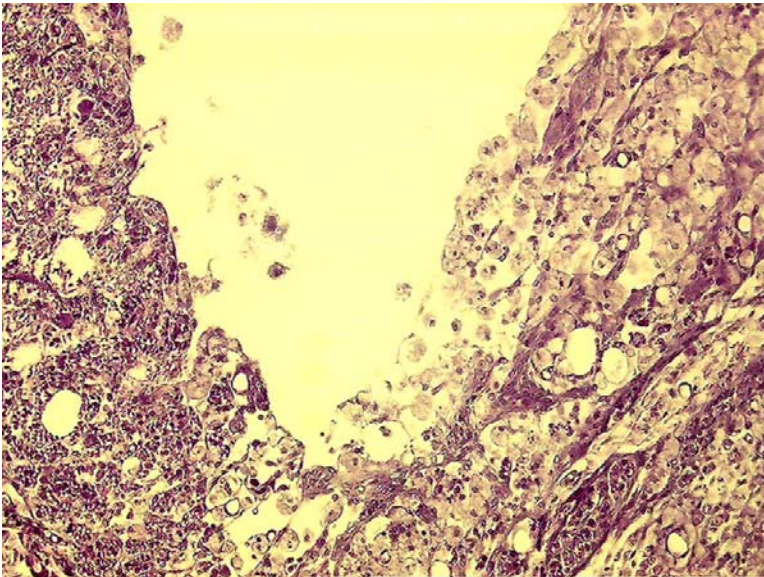


FIGURE 6.3: BBB scores second 1 and 4 weeks after mid-thoracic spinal cord injury with 40 g clip (second experiment). 10-day treatment with 25 μmol/kg quercetin, twice a day. Depicted are means ± SDs. There is a significant difference in BBB scores between treatments with 25 and 2.5 μmol/kg quercetin. There is also significant difference between 25 μmol/kg and saline controls ($p < 0.5$, ANOVA with post-hoc Tukey test).

Iron Localization: Histochemical analysis for iron was performed at 24 hr and 4 weeks after injury in the spinal cords of animals treated for 4 days with either quercetin or saline vehicle (Fig. 6.4). Mallory's iron stain was negative in tissue from all animals sacrificed 24 hr after injury, in quercetin-treated animals as well as in those who received saline vehicle only. In tissue from animals which survived 4 weeks after the trauma, Mallory's iron stain demonstrated macrophage-like cells positive for ferric iron in tissue from the core of the spinal cord damage in all the saline-treated controls, but no iron was detectable in the tissues of animals treated with quercetin (all therapeutic groups).



A: saline-treated control



B: quercetin-treated

FIGURE 6.4: Representative micrographs of spinal cord sections at level of the lesion 4 weeks after spinal cord injury stained for iron using Mallory's stain (iron stains blue). A. Saline-treated animal. B. Animal treated with quercetin (5 $\mu\text{mol/kg}$) for 4 days. Iron-containing (green-blue) cells are clearly seen in the spinal cords of the saline vehicle-treated but not in the quercetin-treated rats.

6.4 Discussion

Our research demonstrates that quercetin administration following spinal cord compression injury promotes the retention of function. In the 4-day quercetin treatment groups one-half of all the animals treated achieved a BBB score of 10 (i.e., occasional weight supported plantar stepping) whereas not one of the saline-treated animals achieved a BBB score greater than 9. In other experiments (Kamencic et al., 2001) we have never seen a saline-treated injured rat achieve a BBB score of greater than 9.

Since most acute traumatic spinal cord injuries do not involve complete transection of the cord, there are two possible approaches to promote function following spinal cord injury: 1) decrease the extent of secondary damage thereby promoting retention of white matter tracts, 2) promote axon regeneration. Our experiments address the first possibility. A number of experimental approaches have been used to decrease secondary damage. Several of these involve decreasing the likelihood of infiltration of inflammatory cells, for example, through monocyte depletion (Popovich et al., 1999), neutrophil depletion (Taoka et al., 1997) or by knocking out cell adhesion molecules required for infiltration of inflammatory cells (Farooque et al., 1999). Although these approaches are of theoretical interest and help delineate the role of inflammatory cells in promoting secondary damage, they are unlikely to be useful therapeutically. More therapeutically likely approaches involve administration of agents that interfere with, for example, activation of cell death cascades such as basic fibroblast growth factor (Rabchevsky et al., 1999; 2000; Cuevas and Carceller, 2001). Other compounds tested include methylprednisolone, tirilazad mesylate, GM1 ganglioside (Constantini and Young, 1994; Koc et al., 1999) and the nitric oxide synthase inhibitor, aminoguanidine (Chatzipanteli et al., 2002). Only few compounds, though, have made it into

clinical trial. The latter group includes tirilazad mesylate, GM1 ganglioside and methylprednisolone. While for the GM1 ganglioside the mechanism of action is not well understood (Nockels and Young, 1992), the major focus of attention for methylprednisolone and the non-glucocorticoid tirilazad mesylate has been the prevention of lipid membrane peroxidation and reduction in inflammatory cytokine production (Braugher and Hall, 1992; Xu et al., 1998; Hall, 2001). There is still considerable controversy regarding the clinical efficacy of these compounds (Fehlings and Bracken, 2001; Hurlbert, 2001).

Based on the study of the toxic effects of hemoglobin on spinal cord neurons in culture, Regan pointed out that release and subsequent degradation of hemoglobin from erythrocytes in the event of hemorrhage after spinal cord injury might contribute to neuronal loss (Regan and Gou, 1998). Indeed, the ferritin upregulation seen after spinal cord injury in humans (Koczyca et al., 2002) supports this notion. Hemoglobin disintegrates to chelatable breakdown products, in the course of which conformational changes occur from the ferrous states of hemoglobin and deoxyhemoglobin into the ferric states of methemoglobin and hemosiderin accompanied by release of some iron ions (Comporti et al., 2002). The formation of methemoglobin with associated iron ion release has been associated with increased activity of lipid peroxidation (Ferrali et al., 1992). Despite the fact that contusional hemorrhage is a common problem with acute traumatic spinal cord injury, none of the compounds tested in a major clinical trial so far has any documented significant capacity to chelate iron, although one of the mechanisms of action of tirilazad mesylate is the inhibition of iron-mediated lipid peroxidation through the scavenging of lipid peroxy (Hall, 1995; Koc et al., 1999). To date, with the exception of one study, no examination of the effect of an iron chelator following spinal cord injury has been performed. The exception is a study

using 2,2'-bipyridine to chelate iron following sectional lesions of the corticospinal tract (Weidner et al., 1999) in order to determine the effect of inhibition of collagen formation on corticospinal tract regeneration; here, 2,2'-bipyridine had no effect on axon regeneration.

As noted in the "Introduction", quercetin has potentially a number of distinct neuroprotective activities. The evidence suggests that one of the more prominent protective activities of quercetin is its ability to chelate iron (Sestili et al., 1998). The MRI results in our experiment indicate that T1 relaxation is a sensitive probe for Fe²⁺-associated protons with iron concentrations ranging from 1 μM to 100 μM, at least in a phantom comprised of agarose and saline. Quercetin was demonstrated to be effective in antagonizing the relaxation effect of T1 proton signal caused by such concentrations of Fe²⁺. The T1 signal induced by 1-10 μM Fe²⁺ could be antagonized by 5 μM quercetin while 100 μM Fe²⁺ could be antagonized by 25 μM quercetin. These observations suggested that the potential therapeutic dose of quercetin could lie between 5 and 25 μmoles quercetin/kg body weight. Indeed, this is what the animal studies indicated. The data demonstrate that treatment with quercetin at doses ranging from 5 to 100 μmol/kg administered 1 hr after injury and then every 12 hr for 4 days produces significantly better recovery from injury than saline vehicle-treatment. When the quercetin dose administered was reduced to 2.5 μmol/kg, no significant therapeutic effect was seen. At the therapeutic dose of 25 μmol/kg no significant differences in outcome was seen whether quercetin was administered twice a day for 4 days or for 10 days.

The therapeutic effect of quercetin could be due to a variety of mechanisms, as outlined in the Introduction. One possibility is that quercetin's therapeutic effect is mediated, in part, through chelation of iron. The 5-25 μmol/kg at which a therapeutic effect was seen is

low in comparison to doses of iron chelators used in other injury model systems, for example 500 $\mu\text{mol/kg}$ 2,2'-dipyridyl in a primate model of subarachnoid hemorrhage (Horky et al., 1998); however, in most of these model systems the chelator was given as a single bolus. That part of the therapeutic effect of quercetin is iron chelation is supported by the fact that the Mallory stain, a modification of the original Perl's stain designed for detection of ferric iron, usually in the form of hemosiderin or ferritin (Thompson, 1966), showed positive cells only in saline vehicle-treated animals 4 weeks after injury, a time point at which it is reasonable to expect the presence of hemosiderin at the site of injury (Bradley, 1993). The ferric iron-containing cells had the morphology of macrophages, which is to be expected by this time following injury (Bradley, 1993).

In summary, the results of our experiments support our working hypothesis that administration of the flavonoid quercetin improves recovery of motor function after acute traumatic spinal cord injury. Further research is required to delineate the therapeutic window and mechanisms by which quercetin exerts its therapeutic effects.

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Chapter 7

Quercetin decreases myeloperoxidase activity and caspase three activation
in an animal model of spinal cord compression injury

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7.1 Introduction

The concept of secondary injury is based on the observation that, hours to weeks after the trauma, cell death by necrosis or apoptosis is induced in cellular structures previously undamaged by the primary mechanical impact, and that damage becomes permanent in tissue structures which potentially could have recovered (Crowe et al., 1997; Shuman et al., 1997; Beattie et al., 2002). Mechanisms contributing to this spreading damage include vascular dysregulation and ischemia, edema formation, traumatic hemorrhage, lipid membrane peroxidation and inflammatory processes (Means and Anderson, 1983; Tator and Fehlings, 1991; Tator and Koyanagi, 1997; Sekhon and Fehlings, 2001; Tator, 2002). It has been suggested that loss of neurological function after spinal cord injury is predominantly caused by loss of functional white matter tracts (Rosenberg et al., 1999). Studies in an animal model of spinal cord compression injury have shown that survival of as little as 10% of all axons in the rat spinal cord is sufficient to support significant motor function (Kamencic et al, 2001; Fehlings and Tator, 1995). Therefore, protection of even a small number of primarily undamaged axons from delayed cell death may result in a considerable difference in functional outcome for the patient. Any compound curbing one or more pathomechanisms of the secondary injury complex should be considered a potentially beneficial compound in the treatment of acute traumatic spinal cord injury, provided its potential adverse effects are negligible.

Quercetin, a well-studied member of the flavonoid family, could be a valuable compound to be tested in this setting. In previous experiments, we have found support for the hypothesis that the capacity of quercetin to chelate iron contributes significantly to the attenuation of oxidative stress after SCI, resulting in improved recovery of motor function

(Schültke et al., 2003a). We have shown that, after application of a clip with 40 g closing force, a high percentage of animals recovered motor function in their formerly paraplegic hind limbs sufficient to support stepping / walking ($BBB \geq 10$), if administration of quercetin was started one hour after the injury. Although iron chelation is one possible explanation for the quercetin-mediated recovery of motor function, more than this one capacity of quercetin appears to be involved in its neuroprotective action. Among the flavonoids studied, quercetin ranks amongst those with the highest anti-oxidative potential (Letan, 1967; Cavallini et al., 1978; van Acker et al., 1995; Zielińska et al., 2001).

The first days after spinal cord injury are characterized by an influx of neutrophils and macrophages into the tissue at the site of injury and adjacent spinal cord segments in both animal model and human patients (Matteo and Smith, 1988; Anderson, 1992; Carlson et al., 1998). Neutrophils, when stimulated, generate potent reactive oxygen species (Hampton et al., 1998; Winterbourn et al., 1985; Badwey and Karnovsky, 1980; Badwey et al., 1991). Superoxide anion radicals ($O_2^{\bullet-}$) are generated by activation of the enzyme NADPH oxidase and subsequently converted to other reactive species including H_2O_2 (Roos, 1991a and 1991b).



The oxidant activity of H_2O_2 is significantly enhanced by the action of myeloperoxidase, a hemoprotein enzyme usually stored in the granules of neutrophils. Myeloperoxidase catalyzes the reaction of hydrogen peroxide and chloride anions, generating hypochlorous acid (HOCl) (reaction 7.2) and chloramines (reaction 7.3) which has a

reactivity about two orders of magnitude higher than that of H₂O₂ alone (Weiss et al., 1982; Thomas et al, 1983; Pincemail et al., 1988; Rodrigues et al., 2002; Gaut et al., 2001).



These oxidant species, together with hydrolytic enzymes, are released into the extracellular space, where they oxidize DNA, proteins and lipids, increasing the volume of secondary damage (Prutz, 1996; Selloum et al., 2001). The extent of tissue damage was reflected in increased formation of protein carbonyls. The extent of carbonyl formation was significantly attenuated in the presence of glutathione. In the setting of spinal cord injury, the increased demand for repair will quickly deplete the cytoprotective glutathione, so that potentially reversible damage becomes permanent (Juurlink and Paterson, 1998). Quercetin has been shown in vitro to decrease myeloperoxidase activity in human neutrophils in a dose-dependent manner, thereby intercepting and limiting the development of pathology just described (Pincemail et al., 1988). Alternatively, quercetin might reduce neutrophil infiltration, which would be reflected in decrease of myeloperoxidase activity at the site of injury. Also, there is evidence that H₂O₂, produced in and released from stimulated human neutrophils, triggers cell death by apoptosis (Lu et al., 2001). Cell death by apoptosis is well known to cause delayed cell death after spinal cord injury in animal model and human patients (Kato et al., 1996; Crowe et al, 1997; Emery et al., 1998). Casha and colleagues (2001) have shown that activation of downstream caspases, such as caspase-3, was directly

proportional to the extent of apoptotic cell death associated with spinal cord injury. Quercetin has been shown to significantly inhibit H₂O₂-induced caspase-3 cleavage and consequent apoptosis in human vascular endothelial cells (Choi et al., 2003). Considering the results from the experiments performed by Lu and colleagues, we would expect to see a decrease in delayed cell death by apoptosis in vivo after administration of quercetin.

Potential actions of quercetin in acute spinal cord trauma might be illustrated as in Figure 7.1.

We have designed this set of experiments to test the hypothesis that administration of quercetin attenuates inflammation and caspase-3 activation as important pathway of apoptotic cell death in the early phase after acute traumatic spinal cord injury.

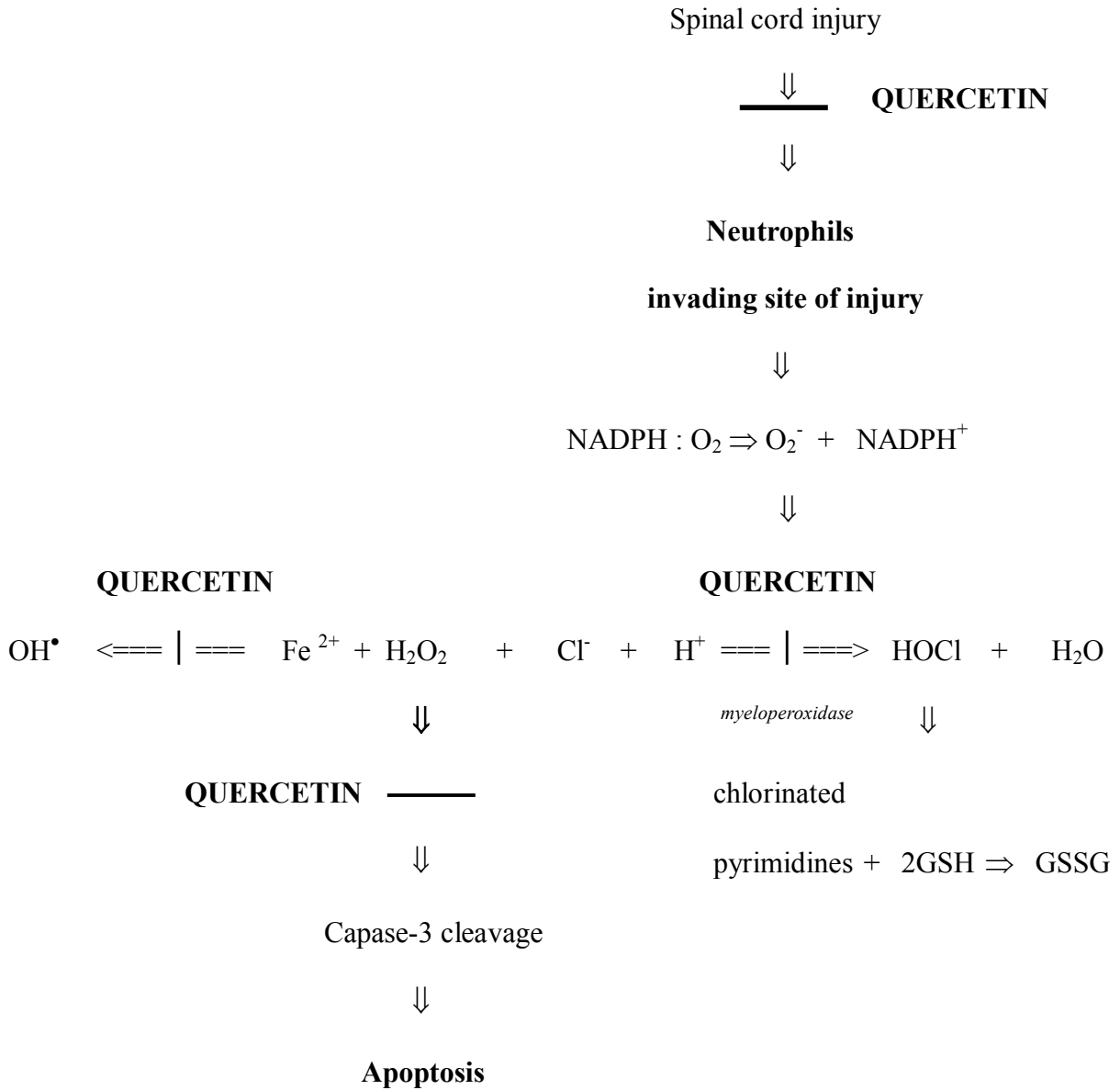


FIGURE 7.1: Quercetin intercepts several pathways involved in creating secondary injury after CNS trauma. The results are decreased production of oxidative species resulting in less demand on the intracellular glutathione pool, and decreased cell death by apoptosis.

7.2 Materials and Methods

A total of 51 adult male Wistar rats (9-10 weeks old, 296-376g, Charles River Canada) were used for the experiments described. Forty-two animals were subjected to standardized mid-thoracic spinal cord trauma (T7). The remaining nine animals were used as uninjured controls. We used the model of mid-thoracic spinal cord compression injury introduced by Rivlin and Tator (Rivlin and Tator, 1978), as described in section 5.3.1.

Animals were assigned to four experimental groups, being sacrificed at 6, 12, 24 or 72 hr after injury (Table 7.1). Half of the animals in each group received doses of 25 $\mu\text{mol/kg}$ quercetin, beginning 1hr after injury and continued in 12 hr-intervals, while the other half of the animals received saline vehicle only. Animals sacrificed 6 hr after injury received only one dose of 25 $\mu\text{mol/kg}$ quercetin dihydrate, 1 hr after injury. Animals sacrificed at 12, 24 and 72 hr after injury received their last doses about 40-50 minutes before they were sacrificed.

survival after injury	6 hr		12 hr		24 hr		72 hr	
	quercetin	saline	quercetin	saline	quercetin	saline	quercetin	saline
BIOCHEMISTRY	3	3	4	4	4	4	4	4
IMMUNOCYTO CHEMISTRY	0	0	3	3	3	3	3	3

TABLE 7.1 Group assignment of experimental animals.

Myeloperoxidase activity was measured in both spinal cord tissue and serum at 6, 12, 24 and 72 hr after injury by the spectrophotometric method as described in section 5.6.

Caspase-3 immunostaining was performed on sections from animals sacrificed at 12, 24 and 72 hr after injury as described in section 5.5 (Table 7.1).

7.3 Results

Myeloperoxidase activity: Significant decrease of myeloperoxidase activity in spinal cord tissue at the site of injury was found with quercetin treatment at 24 hr ($p = 0.01$) after trauma (Fig. 7.2). No statistically significant difference was seen at 6, 12 and 72 hr after injury. No significant myeloperoxidase activity was detected in the spinal cord segments cranial (T4) or caudal (T10) from the injury site.

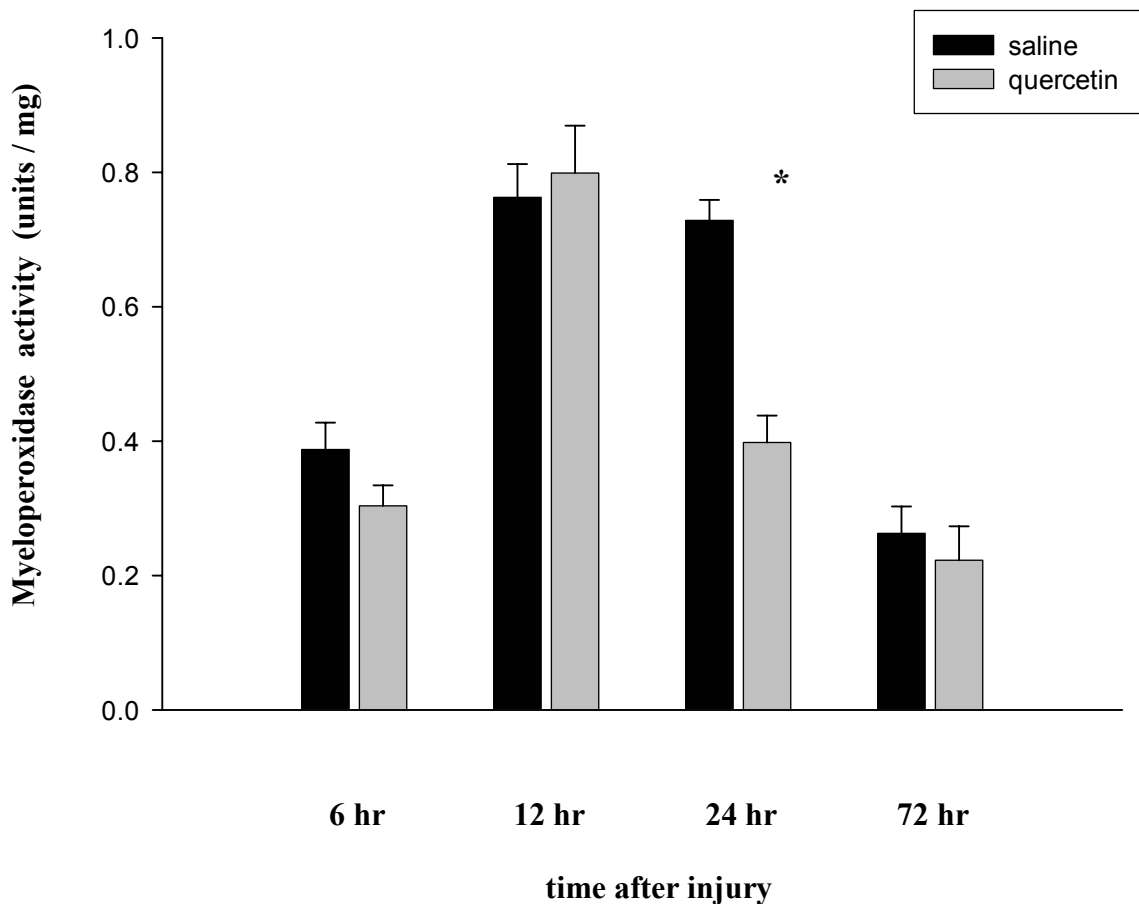


FIGURE 7.2: Myeloperoxidase levels at the site of injury after 50 g clip injury in the adult male Wistar rat. Significantly lower MPO levels seen only at 24 hr after injury. Depicted are Means \pm SD.

In plasma, significantly reduced levels of myeloperoxidase activity is seen at 6 hr ($p = 0.02$), 12 hr ($p = 0.04$) and 24 hr ($p = 0.04$) with administration of quercetin (Fig. 7.3). Although there were lower MPO activity levels at 72 hr after injury, this was not statistically significant.

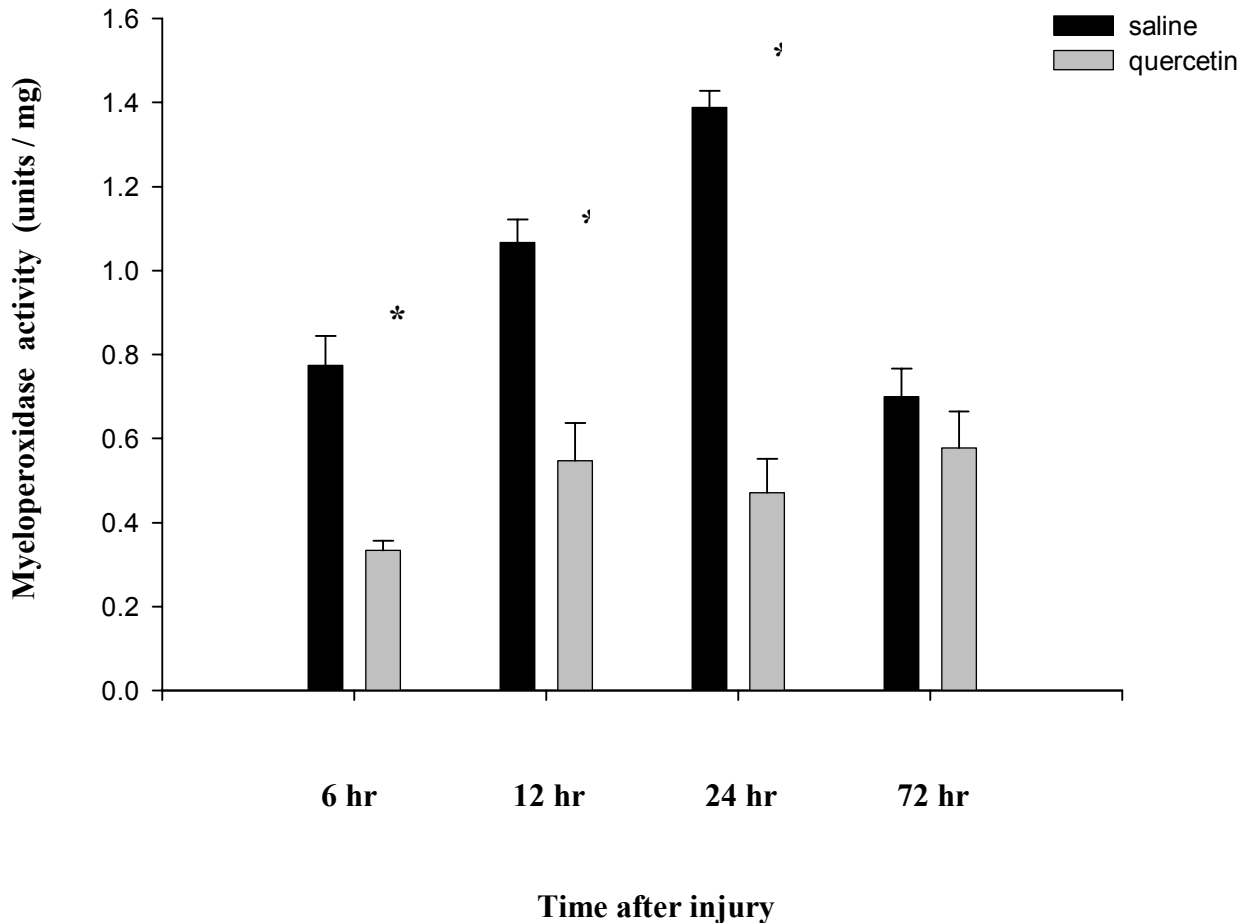


FIGURE 7.3: Myeloperoxidase levels in plasma of injured and healthy animals.

Significantly lower myeloperoxidase levels are seen at 6, 12 and 24 hr after injury. Depicted are Means \pm SD.

Myeloperoxidase was almost not detectable in spinal cord tissue and serum of healthy, uninjured animals.

Activated caspase-3 expression: Activated caspase-3 was found in a small proportion of glial (mostly astroglial) cells in the spinal cord sections of uninjured animals (Figs. 7.4 A and 7.5 A). After injury, massive necrosis was seen at the site of injury. Activated caspase-3 expression was therefore assessed in the segments immediately cranial and caudal to the site of injury. No obvious increase of activated caspase-3 expression was seen at 12 and 24 hr after injury in either quercetin-treated animals or saline controls. However, at 72 hr after injury, a large increase in the number of caspase-3-positive cells was noted in saline controls, both in neurons and glia (Figs. 7.4 B and 7.5 B). In the tissue of quercetin-treated animals, however, there was only very little increase in activated caspase-3 expression, when compared to sections from healthy, uninjured spinal cords (Figs. 7.4 C and 7.5 C).

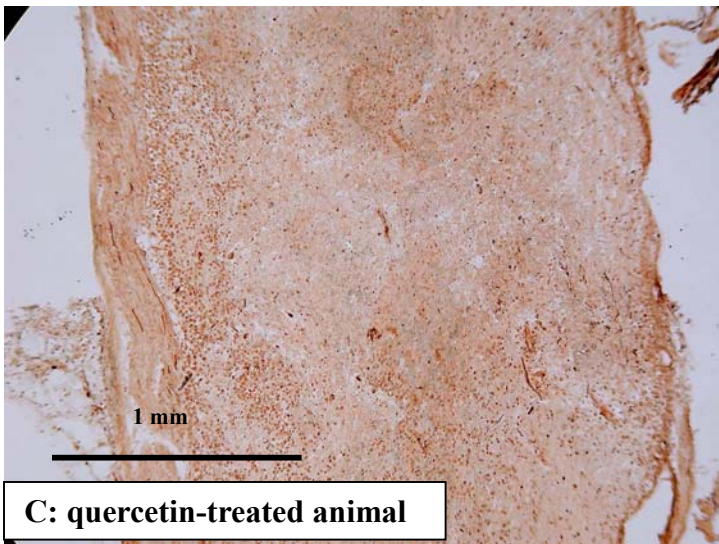
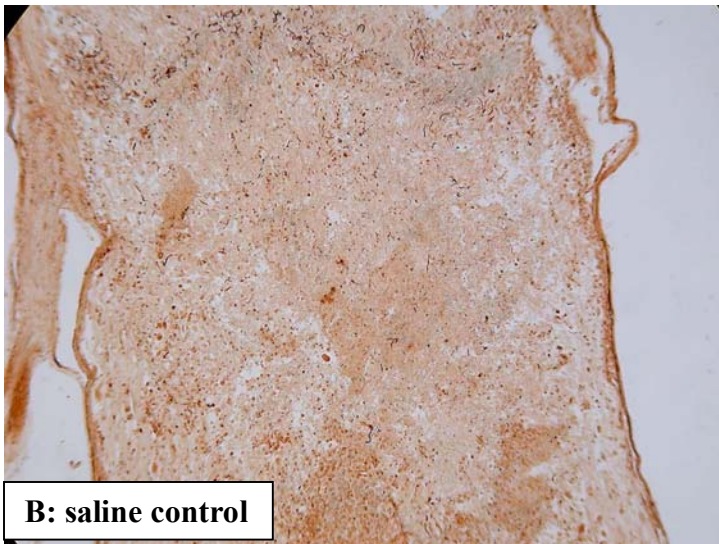


FIGURE 7.4:
Activated caspase-3 immunostaining.
Spinal cord of adult Wistar rat, 72 hr after
50 g clip application for 5 sec.
Low magnification. Center of injury site.

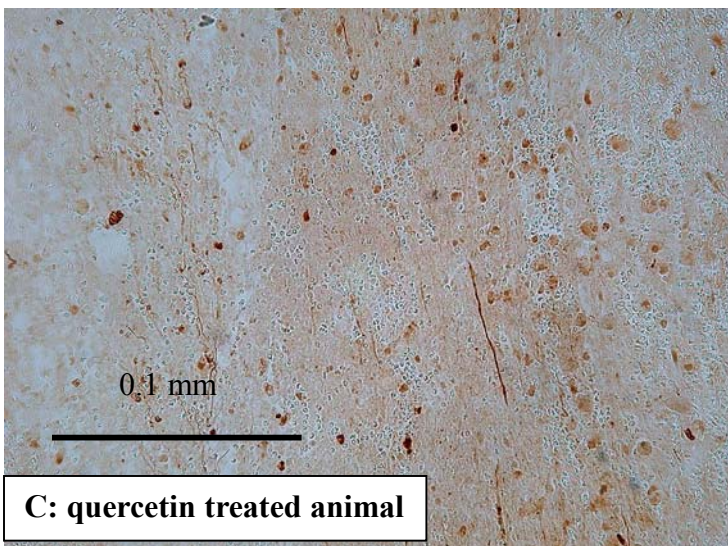
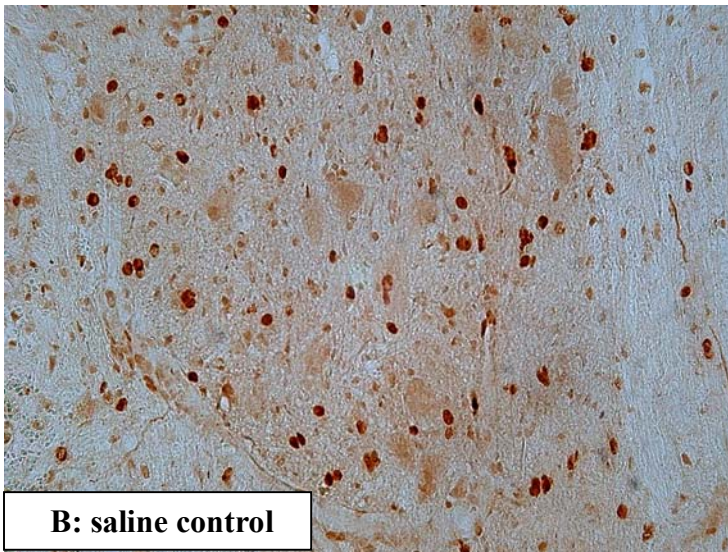
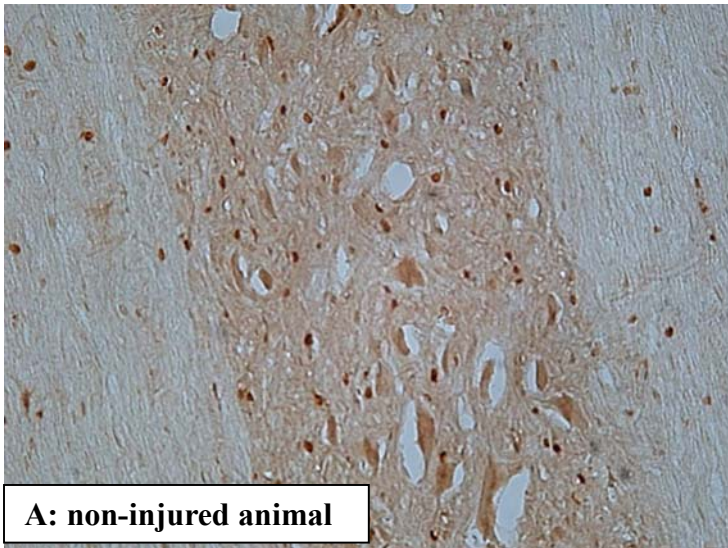


FIGURE 7.5:
Activated caspase-3 immunostaining.
Spinal cord of adult Wistar rat, 72 hr after
50 g clip application for 5 sec.
High magnification.
Periphery of injury site.

7.4 Discussion

The results of our experiments show that twice daily administration of 25 $\mu\text{mol/kg}$ quercetin significantly reduces inflammation and suggest that apoptosis is reduced at the injury site in male adult Wistar rats, when treatment is begun 1 hr after injury.

Since inflammatory processes have been shown to contribute to increase of lesion volume after physical injury, it seems reasonable to test compounds with inflammatory capacity, to inhibit the deleterious cascade of secondary injury (Simpson et al., 1991; Juurlink and Paterson, 1998; Eng and Lee, 2003). Neutrophil activity has been used by other researchers to demonstrate the anti-inflammatory capacity of drugs tested for its therapeutic potential in animal models (Tonai et al., 2001; Hirose et al., 2000; Hara et al., 2000; Fujimoto et al., 2000; Taoka et al., 1997b). Myeloperoxidase activity has been shown to reflect fairly well the extent of neutrophil accumulation in tissue (Bradley et al., 1982). The enzyme myeloperoxidase, stored in the granules of neutrophils, has been described as a key regulator in the oxidant production by cellular mediators of inflammation (Kettle and Winterbourn, 1997). While measurement of protein carbonyl content offers an indirect method to assess the extent of cellular protein reaction with HOCl (Chapman et al., 2000), most commonly the capacity of myeloperoxidase to form hypochlorous acid from hydrogen peroxide and chloride ions is used. Carlson and colleagues have assayed myeloperoxidase activity in the spinal cord of rats between 4 and 48 hr after acute spinal cord contusion injury (Carlson et al., 1998). They found that MPO activity peaked at 24 hr after injury, with the center of activity found between 4 mm rostral and caudal to the site of injury. While the localization of significant myeloperoxidase activity in this study corresponds with our findings, we detected maximum MPO activity already at 12 hr after the injury, a time point not examined by

Carlson and colleagues. The reduction in myeloperoxidase activity seen with administration of quercetin in our experiments indicates that modulation of neutrophil activity is one of the pathways through which this compound acts in the setting of spinal cord injury. The finding that there is significant reduction of myeloperoxidase activity in the plasma of injured animals with administration of quercetin suggests that quercetin is inhibiting the activation of neutrophils and that the decreased MPO activity in injured spinal cords is likely due to decreased infiltration of neutrophils. Furthermore, the inhibition of myeloperoxidase activity in plasma reflects a general anti-inflammatory activity of quercetin, rather than one specific for the CNS. This might be due to differences in the injury models used. The reduction in myeloperoxidase activity seen with administration of quercetin in our experiments indicates that modulation of MPO activity is one of the pathways through which the compound acts in the setting of spinal cord injury. This notion is supported by significant reduction of myeloperoxidase activity in the serum of injured animals with administration of quercetin. Whether this is due to an inhibition of neutrophil influx or an inhibition of the enzyme's activity, remains to be investigated. Since neutrophil activation after trauma is not specific for CNS injury, the inhibition of myeloperoxidase activity in serum reflects a general anti-inflammatory activity of quercetin, rather than one specific for the CNS.

Experiments performed in our laboratory suggest that there is a beneficial effect with administration of quercetin for three days or longer (Schültke et al., 2003b). A statistically significant difference was seen in myeloperoxidase levels at the injury site at 24 hr, but not at 72 hr after injury. Yet, a significant improvement in recovery of motor function was seen when the duration of quercetin administration was increased from 24 hr to 3 days. This suggests that the functional improvement with quercetin administration beyond 24 hr must be

conveyed through a pathway other than inhibition of myeloperoxidase activity. From the results of our experiments here presented we conclude that inhibition of apoptosis could be another such pathway through which quercetin acts neuroprotective. It has been described earlier that disintegration products of hemoglobin contribute to oxidative stress and apoptosis in the CNS (Matz, 2000; Gaetani, 1998). Our finding that there was less increase in activated caspase-3 expression with quercetin treatment suggests that cell death by apoptosis is inhibited in vivo just as in Lu's in-vitro experiments. Notable here is the fact that significant increase in apoptosis was not seen before three days after injury in untreated animals, which coincides with the time course when hemoglobin disintegration would be expected. Katoh and colleagues (1996) have studied apoptotic cell death in adult rats, using a model of spinal cord compression injury. Although different analysis techniques (DNA laddering and in situ end labeling) were used to detect apoptosis, they found that the maximum of fragmented cells was seen at three and four days after injury. This would correspond with our findings that a significant increase of caspase-3 expression was observed at 72 hr after injury. This increase of activated caspase-3 expression was apparently prevented by quercetin administration. It has been demonstrated in several cell culture models, that quercetin reduces H₂O₂-induced apoptosis (Choi et al., 2003; Park et al., 2003). A similar mechanism might account for the compound's anti-apoptotic activity in our in-vivo model. Wang and colleagues were able to demonstrate in cultures of kidney epithelial cells that H₂O₂-induced apoptosis was accompanied by c-Jun N-terminal kinase (JNK) activation (Wang et al., 2002). JNK expression was also found to be increased by the action of 4-hydroxy-2-nonenal (HNE), a lipid peroxidation-derived product from arachidonic or linoleic acid in the setting of oxidative stress (Uchida et al., 1999). Experiments reported in the same paper showed that

quercetin exerted a significant inhibitory effect on (HNE)-induced JNK activation. Thus, it is a distinct possibility that the anti-apoptotic effect of quercetin in our spinal cord injury model is conveyed through inhibition of JNK. Since delayed cell death by apoptosis is an important mechanism in the generation of secondary injury volume, we believe that the inhibition of apoptotic cell death is another important mechanism by which quercetin acts in a neuroprotective fashion.

Curiously, we noted that a proportion of astrocytes in uninjured spinal cord tissue exhibited activated caspase-3 in their nuclei. It is known that caspase-3 has non-apoptotic functions (Schwerk and Schulze-Osthoff, 2003).

In summary, we have demonstrated that quercetin decreases the activity of activated neutrophils, as reflected in decrease of myeloperoxidase activity in both plasma and spinal cord tissue at the site of injury in the setting of acute traumatic spinal cord injury in a rat model. The decrease of activated caspase-3 expression strongly suggests that administration of quercetin results in a decrease of apoptotic cell death of neurons, compared to animals that received saline vehicle only. These results suggest that administration of quercetin does decrease secondary tissue damage after acute spinal cord trauma.

Quercetin is present in many components of our diet (de Vries et al., 1996; Hollman and Katan, 1999), is readily taken up after oral intake (Hollman et al., 1996; Moon et al., 2000) and can attain plasma concentrations of 1 μ M. The fact that quercetin is widely distributed in the diet and is, therefore, a compound with which we have evolved, suggests it would be a safe drug to use for therapeutic intervention. We have seen no adverse effects when quercetin was administered to rats in doses as high as 200 μ mol /kg/day (Schültke et al., 2003). This is

in keeping with other research indicating that rats can readily tolerate long-term intake of 1.5 mmoles quercetin/ kg/day (IARC, 1999).

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Chapter 8

Neuroprotection after head trauma:

A pilot study of quercetin with the fluid percussion model

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8.1 Introduction

With increasing numbers of head trauma survivors in most parts of the Western world over the last decades, the search for measures to prevent secondary injury once prevention of primary injury has failed is becoming a major focus of attention. In earlier experiments, we have been able to demonstrate in an animal model of acute traumatic spinal cord injury the neuroprotective effect of the polyphenolic flavonoid quercetin (Schültke et al., 2003). Administration of 25 $\mu\text{mol/kg}$ quercetin twice daily, beginning one hour after injury, resulted in significantly improved recovery of motor function in paraplegic animals. The objective of the present study was to determine whether a similar protective effect could be achieved in an animal model of moderate head trauma. For our experiments, we used the fluid percussion model developed by McIntosh and colleagues (1989), as described in section 5.3.2.

One important pathway, through which quercetin apparently acts as a neuroprotectant *in vivo*, is the inhibition of oxidant production by activated neutrophils (Pincemail, 1988; Lu et al., 2001; Zielińska et al., 2001). Some of the neuropathological consequences seen in survivors of acute head trauma have been attributed to post-traumatic inflammatory response in the traumatized brain (Holmin et al., 1998). Inflammation is associated with the influx of neutrophils into the site of injury, in both human patients and animal model (Schoettle et al., 1990; Holmin et al., 1998). Activated neutrophils generate potent oxidant species, which they release into the extracellular space (Badwey and Karnovsky, 1980; Pincemail, 1988; Badwey et al., 1991). There, they will react with healthy tissue structures bordering the area of primary tissue injury. Hydrogen peroxide (H_2O_2), one of the reactive oxygen species released by stimulated neutrophils, reacts with ferrous iron ions (Fe^{2+}) to create hydroxyl radicals (HO^\bullet), a strong oxidant species. The reactive potential

of hydrogen peroxide is increased 100-fold by the action of the enzyme myeloperoxidase within the neutrophils (Pincemail et al., 1988). Myeloperoxidase is a hemoprotein enzyme stored in the granules of neutrophils. It catalyzes the oxidation of chloride anions (Cl⁻) by hydrogen peroxide, thereby generating hypochlorous acid (HOCl) and chloramines (Weiss et al., 1982; Thomas et al., 1983). These chlorinated species act as strong oxidants themselves. When released from the neutrophils, they contribute to the destruction of healthy tissue bordering the primary site of injury thereby significantly increasing the lesion volume of the secondary tissue injury. Secondary damage is created by oxidative damage to phospholipid membranes. In axons, integrity of membrane function is reflected by the amplitude and duration of action potentials. Therefore, we chose measurements of Compound Action Potentials after moderate fluid percussion injury to the brain as primary component for analysis in our study, to investigate whether quercetin acted indeed neuroprotective in the setting of traumatic head injury. Additionally, measurements of myeloperoxidase activity and glutathione levels in the injured brain tissue as well as tissue culture experiments, demonstrating the effect exerted by quercetin administration on the extracellular pH, were performed to elucidate possible mechanisms of this neuroprotective effect. ANOVA with post hoc Tukey test was used for statistical data analysis.

8.2 Material and methods

Out of 34 adult male Sprague Dawley rats (57 – 68 days old, 285 – 366 g; Charles River Canada), 26 animals were submitted to moderate fluid percussion injury as described in section 5.3.2 (Table 8.1). Injured animals were divided into two groups: one group received 25 $\mu\text{mol/kg}$ quercetin intra-peritoneally, starting 1 hour after injury with continued treatment in 12-hr intervals, while animals in the second group received weight-adjusted doses of normal saline according to the same schedule (n = 13 per group). Out of each group, eight animals were sacrificed 24 hr after injury, and five animals were sacrificed 72 hr after injury by Halothane® overdose and decapitation. All animals received their last injections about one hour before sacrifice. While animals used for electrophysiology experiments were beheaded and brains immediately extricated, animals used for histological and biochemical analysis were sacrificed by pericardiac perfusion with ice cold saline.

Sacrificed at	Quercetin		Saline		Non-injured	
	CAP	Histology	CAP	Histology	CAP	Histology
24 hr after injury	5	3	5	3	5	3
72 hr after injury	5	0	5	0		

Table 8.1: Animals used for fluid percussion injury.

Twenty animals (10 quercetin-treated and 10 receiving saline vehicle alone) were used to determine the post-traumatic amplitudes of compound action potentials (CAP) in the

corpus callosum. For this purpose, slices of 400 μm thickness, were cut on the vibrotome from the brain tissue that received the maximum impact of the fluid percussion injury. CAP acquisition followed the procedure described in section 5.3.2. CAP of injured animals were compared with CAP of 5 healthy control animals.

To correlate structural damage and some parameters of anti-oxidative, neuroprotective state in the brain section which received the maximum impact from the fluid percussion injury, the brains of 6 animals (3 quercetin-treated and 3 saline controls) were available. These six animals were sacrificed 24 hr after injury. The brains of three healthy, uninjured animals were used as controls. All brains used for biochemical analysis were snap-frozen in liquid nitrogen. The area absorbing the majority of the kinetic energy of the impact in the experimental groups was divided into 4 sectors (Figure 8.1). The brains were then split longitudinally along the midline. The two sectors of the right hemisphere were processed for histological studies, while the sectors of the left hemisphere were processed for biochemical analysis.

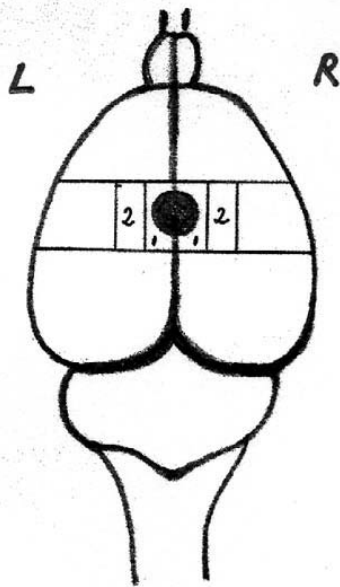


FIGURE 8.1: Schematic showing the point of impact (black dot) with the fluid percussion injury model, and the sectors (L 1 and 2, R 1 and 2) of brain tissue analyzed.

R: right hemisphere, processed for histology

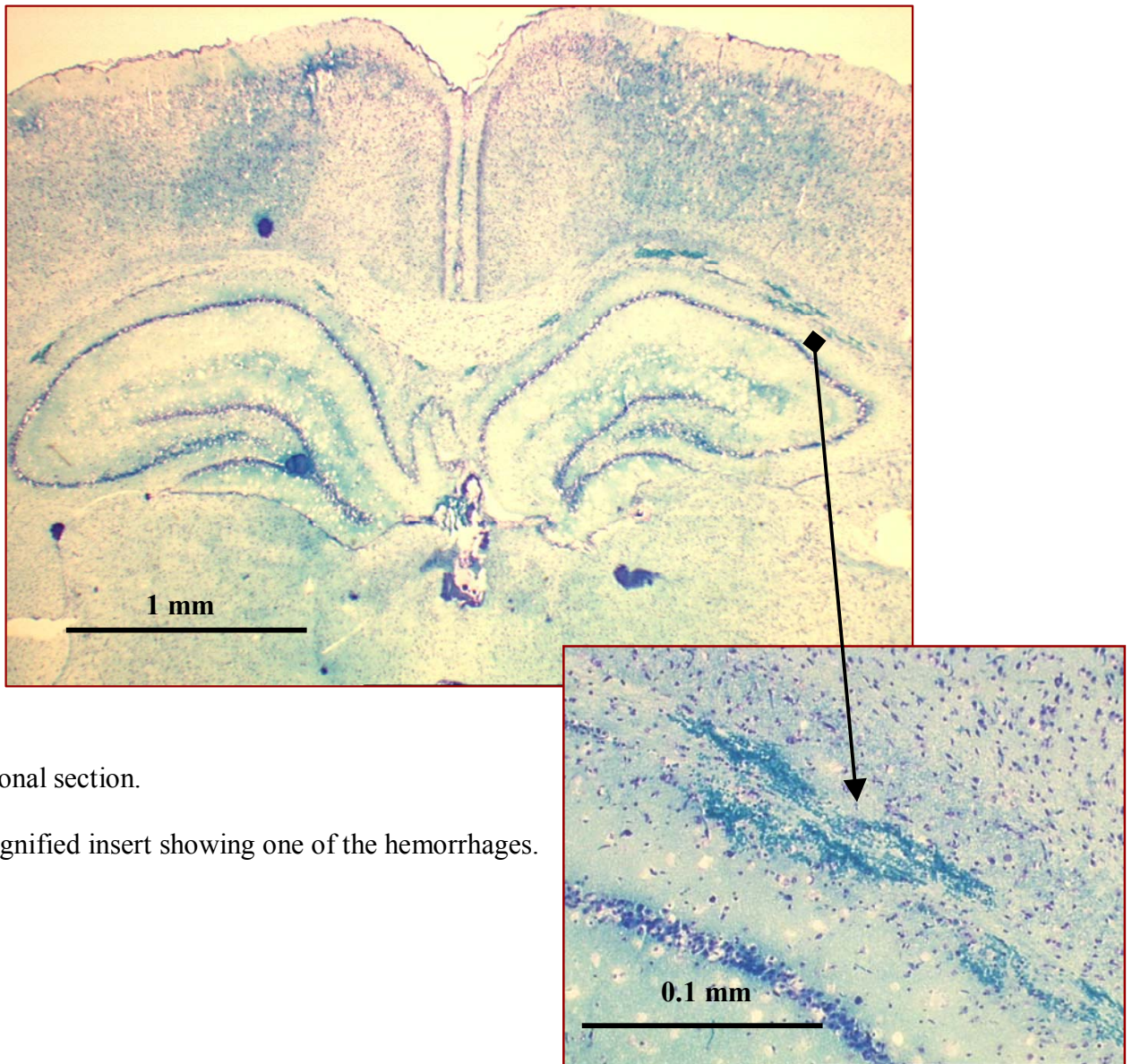
L: left hemisphere, processed for biochemical analysis

Histology: Frozen sections of 20 μm thickness were mounted on gelatin-coated glass slides (Colorfrost, VWR). Alternating sections were stained with Hematoxylin & Eosin (H&E) and Luxol Fast Blue (LFB) -Cresylviolet stain.

Biochemistry: The levels of myeloperoxidase activity were measured as marker of post-traumatic inflammation. Glutathione content was used as marker for tissue protective status; it was determined in brain tissue samples at 24 hr after injury, using the procedures described in section 5.6.

8. 3 Results

Histology: Diffuse petechial hemorrhages were found throughout the corpus callosum of traumatized brains of all animals, in quercetin-treated animals as well as in those receiving saline vehicle only (Figure 8.2).



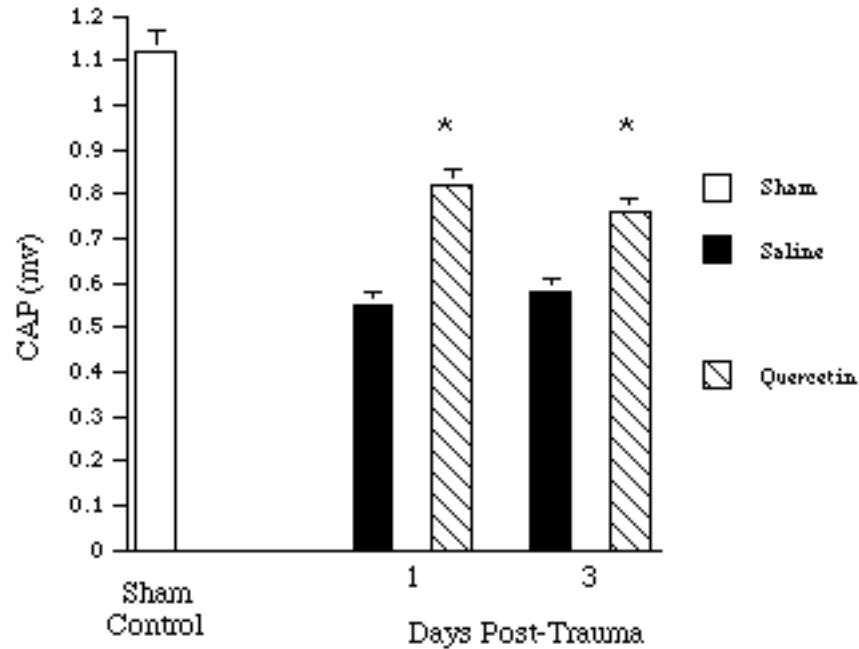
Coronal section.

Magnified insert showing one of the hemorrhages.

FIGURE 8.2: Hemorrhages in corpus callosum of adult male Wistar rat, 24 hr after moderate fluid percussion injury. LFB – Cresylviolet stain.

Electrophysiology: At both 24 hr and three days after moderate fluid percussion injury, compound action potentials (CAP) were found to be significantly higher in quercetin-treated animals, i.e. closer to the values expected from healthy, uninjured animals, when compared with saline controls (Figure 8.3). While in non-injured, healthy animals CAP amplitudes were found between 1 and 1.2 mV, the amplitude dropped for injured saline controls animals to between 0.51 and 0.58 mV. With quercetin treatment, amplitudes were between 0.76 and 0.85 mV, which was significantly higher than for animals that received saline vehicle only. Three days after injury, CAP had recovered somewhat even in saline controls, to a range between 0.56 and 0.62 mV. CAP amplitudes measured in animals that received quercetin were between 0.72 and 0.78 mV. The amplitudes measured in quercetin-treated animals at three days after injury are slightly (albeit not statistically significant) lower than those measured 24 hr after injury, amplitudes are still significantly higher than in animals that received saline vehicle only (Figure 8.3).

Compound Action Potentials In Corpus Callosum Following Moderate Fluid Percussion Injury



* P<0.001, Quercetin versus Saline, ANOVA with post hoc Tukey test

FIGURE 8.3: Compound Action Potentials (CAP) at 1 and 3 days after moderate fluid percussion injury in the adult Sprague Dawley rat. Treatment with the anti-inflammatory and antioxidant compound quercetin results in significantly improved action potentials, compared to saline controls. Depicted are Means \pm SD.

Biochemistry:

Myeloperoxidase activity: Myeloperoxidase activity levels in the samples taken from the center of the injured brain tissue (sector 1) at 24 hr after injury were found to be significantly lower in quercetin-treated animals when compared with saline ($p < 0.05$). A tendency towards lower myeloperoxidase activity compared to saline-treated animals was observed also in the samples taken more lateral from the center of the injury (sector 2), but this was not statistically significant (Figures 8.4 and 8.5).

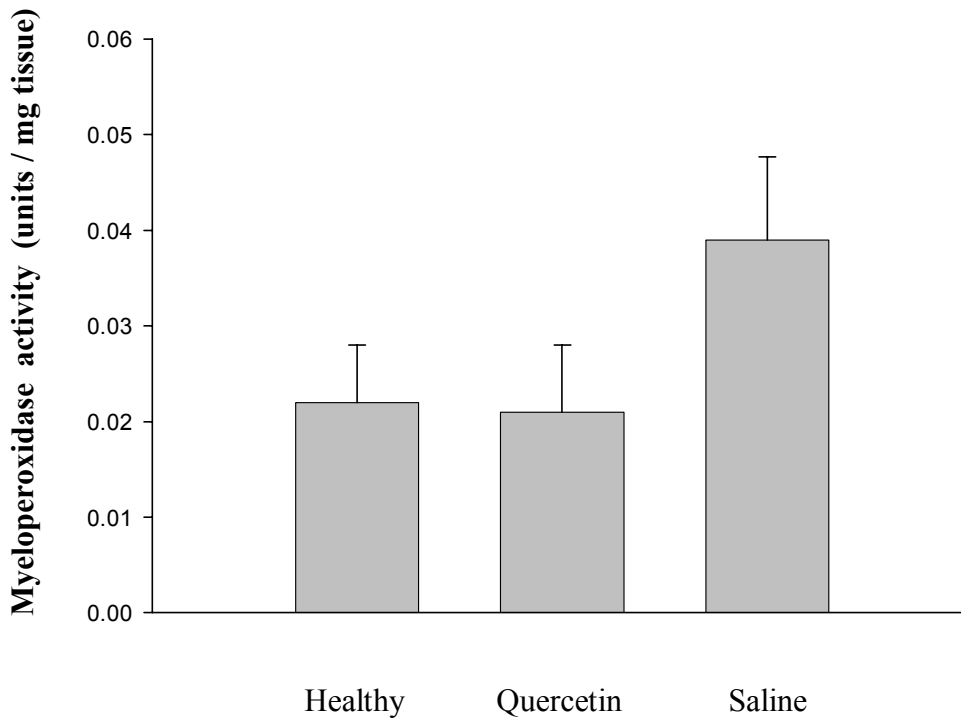


FIGURE 8.4: Myeloperoxidase levels in brain samples taken from center of impact (1), 24 hr after moderate fluid percussion injury in the adult male Sprague Dawley rat. Depicted are Means \pm SD.

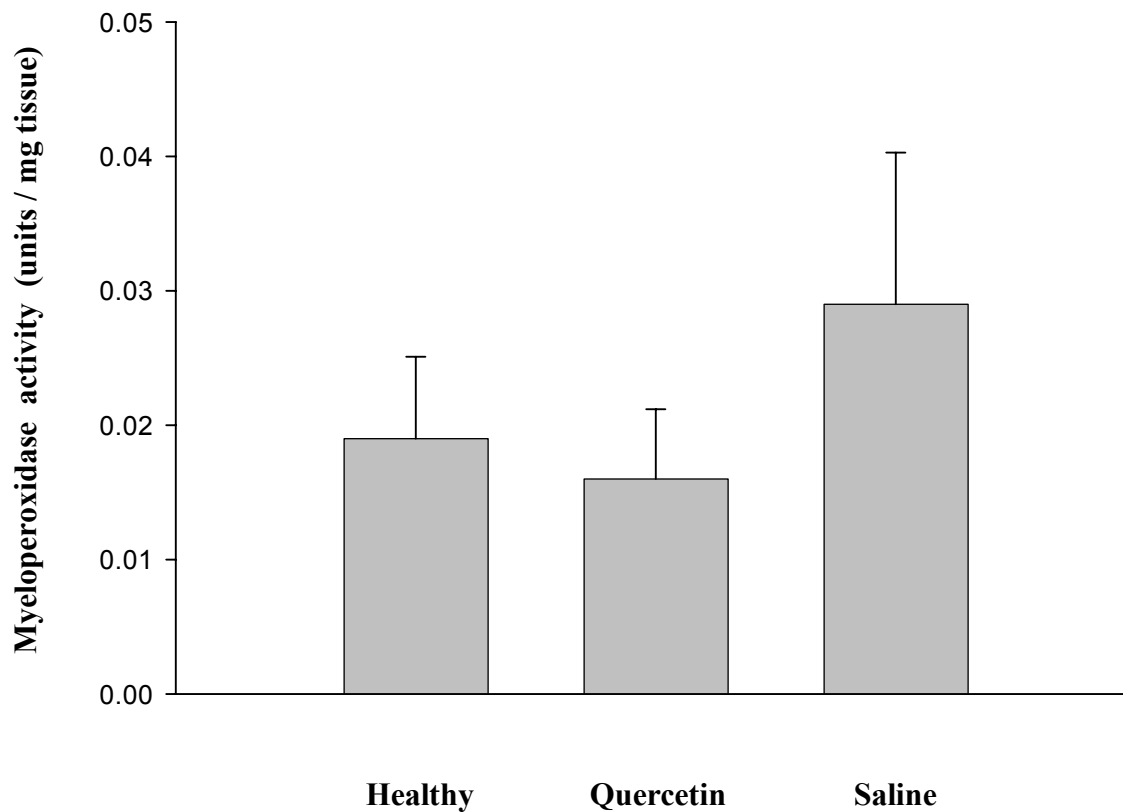


FIGURE 8.5: Myeloperoxidase levels in brain samples taken laterally from center of impact (2), 24 hr after moderate fluid percussion injury in the adult male Sprague Dawley rat. Depicted are Means \pm SD.

GSH levels: The levels of glutathione (GSH) at 24 hr after fluid percussion injury were found to be significantly higher in samples taken from the center of the injured brain tissue (1) of quercetin-treated animals, when compared to those of saline controls ($p = 0.024$). A tendency towards higher glutathione levels was found in the samples taken laterally from the center of injury (2), but there was no statistical significance (Figure 8.6).

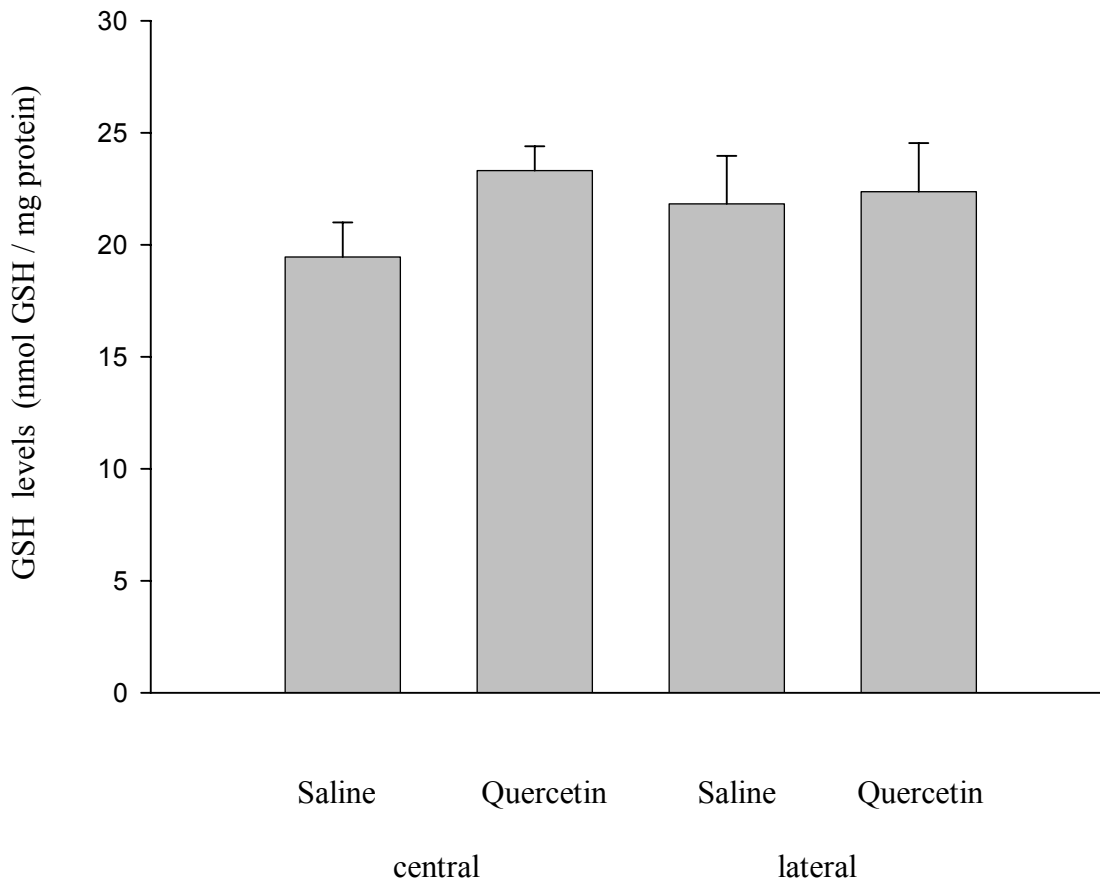


FIGURE 8.6: Levels of GSH in samples taken from central and lateral sectors of brains after moderate fluid percussion injury (quercetin-treated vs. saline controls). Depicted are Means \pm SD.

8.4 Discussion

The animal model of fluid percussion injury to the brain can be clinically equated best to brain concussion in human patients. Similar pathological processes can be detected in the animal model and in human patients with concussion (Hovda et al., 1995). Although the pathology summarized under the term “brain concussion” originally was thought to comprise only temporary disturbances of brain function caused by neuronal, chemical, or neuroelectrical changes, it is now well known that structural damage can occur in this setting (Jane et al., 1985; Cantu, 2000). Concussion is characterized by injury of diffuse nature due to biomechanical forces acting on the brain, and a relative paucity of histologically detectable damage (Giza and Hovda, 2000). No consistent neuropathologic equivalents have been found in experimental models of cerebral concussion. However, capillary damage, transient cerebral ischemia, edema, widespread neuronal depolarization from acetylcholine release, and shearing of neurons and axons as potential mechanisms for the alteration in mental status have been seen after closed head injury (Warren and Bailes, 2000; Graham, 1996). A frequently encountered consequence of head trauma is diffuse axonal injury (Gentleman et al., 1995; Maxwell et al., 1997). Furthermore, lipid membrane peroxidation following oxidative stress and inflammatory processes superimposed on the mechanically damaged CNS tissue result in significant increase of lesion volume. The extent of this secondary injury is positively correlated with outcome after injury (Miller and Becker, 1982). The results from experiments performed in other laboratories suggest that myeloperoxidase activity levels are positively correlated to oxidative stress, inflammatory status and integrity of axonal membranes. Barone and colleagues, correlating histology (H&E and histochemical staining for leucocytes) and myeloperoxidase assay in a rat model of cerebral focal ischemia,

demonstrated that the increase of myeloperoxidase seen in brain after the ischemic injury was a selective function of polymorphonuclear leucocytes (PMN) (Barone et al., 1991). In our experiments, we found that myeloperoxidase activity was positively correlated to post-traumatic loss of neuronal function, expressed in the amplitude of compound action potentials. When Pincemail and colleagues studied the effect of flavonoids on activity of human neutrophil activity in vitro, they found that, among the flavonoids investigated, quercetin was the most potent inhibitor of myeloperoxidase (Pincemail et al., 1988). The fact that administration of quercetin in our model resulted in decreased myeloperoxidase levels, therefore, supports the idea that quercetin acts neuroprotective partly through its antioxidant and anti-inflammatory action. The fact that, with quercetin administration a statistically significant decrease of myeloperoxidase activity is seen at 6, 12 and 24 hr in plasma, yet only at 24 hr in tissue after spinal cord injury could be due to an inhibition of activity of circulating neutrophils. Reduced activity in plasma suggests reduced neutrophil activation in the first place. If we understand myeloperoxidase activity in the tissue as accumulated over time, while myeloperoxidase activity in plasma is based on the number of activated circulating neutrophils alone, our results would suggest that 1) neutrophil invasion at the site of injury is reduced and that 2) quercetin interferes with degranulation of myeloperoxidase rather than chemically inhibiting its action.

Oxidative damage to lipid membranes in CNS structures will effectively result in loss of function, which can be perceived as functional axotomy. It has been suggested that axotomy is a consequence of impaired axoplasmatic transport (Povlishock and Christman, 1995). The results of our experiments suggest that quercetin reduces the extent of secondary functional axotomy caused by oxidative stress and inflammatory processes.

It has been demonstrated in cell cultures that quercetin significantly inhibits the phosphorylation of c-Jun N-terminal kinase (JNK) caused by the action of 4-hydroxy-2-nonenal (HNE), a lipid peroxidation-derived product from arachidonic or linoleic acid in the setting of oxidative stress (Uchida et al., 1999). In the same paper, it was shown that quercetin prevented HNE-induced depletion of GSH. Increasing glutathione is an effective way to protect CNS tissues against oxidative stress-induced damage, as reviewed by Juurlink and Paterson (1998). Our observation that glutathione levels were higher in the brains of quercetin-treated animals therefore attests to a higher cytoprotective status at the site of injury in those animals.

Levels of GSH or ratios of reduced to oxidized glutathione (GSH / GSSG ratios) have been used to describe the cytoprotective potential of cells in the setting of oxidative stress. Decreased intracellular GSH levels have been associated with the oxidation of regulatory proteins, a process reversible by the re-establishment of normal GSH levels (Gilbert, 1990; Sen, 2000). It has been shown in a model of acute GSH depletion in cardiac muscle cells that decrease in intracellular GSH levels was associated with significant decrease in duration of action potentials, decrease of rise in the depolarization phase and a slight, although not statistically significant decrease in the amplitude of action potentials (Pacher et al., 1998). Histological analysis done in this study revealed ultrastructural alterations including intracellular and interstitial edema, swelling of the mitochondria, a decrease of mitochondrial matrix density and rupture of cristae in the mitochondria as consequence of glutathione depletion. Since CNS trauma is followed by increased production of free radicals in the tissue, GSH levels are expected to drop early after injury (Juurlink, 1999). Therefore, changes in the size of action potential are to be expected in the setting of head trauma. Our experiments have shown that moderate fluid percussion injury resulted in both decreased

GSH content and loss of action potential amplitude, while quercetin administration improved (or partially preserved) GSH levels and action potentials. One possible explanation for the preservation of the GSH pool with quercetin administration is that quercetin reduces the amount of oxidants produced after injury. In this case, the higher glutathione levels found in brain samples from quercetin-treated animals would reflect preservation of the GSH pool rather than repletion through quercetin. No histological analysis on electronmicroscopic level was done in our study. However, considering the mitochondrial damage after loss of intracellular GSH observed in the previously mentioned cardiac muscle study, our results suggest that, in the setting of head trauma, preserved mitochondrial function might contribute significantly to membrane integrity and therefore to its capacity to mount adequate action potentials.

Thus, our findings suggest that administration of quercetin after head trauma is neuroprotective and preserves axonal function.

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Chapter 9

Finding the therapeutic window for administration of quercetin after acute traumatic spinal cord compression injury in the adult rat

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Submitted to J Neurosurgery

9.1 Introduction

In earlier experiments, we have demonstrated that administration of quercetin after spinal cord injury in a rat model of spinal cord compression injury decreased myeloperoxidase activity at the site of injury (Schültke et al., 2002). Quercetin is also believed to decrease oxidative stress by chelating free iron ions (Morel, 1993; van Acker, 1995; Afanas'ev et al., 1988; Romanova, 2001). Results from our earlier experiments suggested that quercetin administration improves iron clearance from the site of injury and contributes to improved recovery of motor function in the setting of spinal cord compression injury in the adult rat (Schültke et al. 2003). Furthermore, quercetin has been found to modulate scar formation in soft tissue (Schültke et al., 2003) after spinal surgery. Finally, we have also shown that quercetin inhibits apoptosis. In order to test our hypothesis, that quercetin administration can be beneficial for recovery of motor function when administered either in the acute phase, or in the post-acute phase of acute traumatic spinal cord injury, we have chosen the animal model of acute traumatic, mid-thoracic spinal cord compression injury in the adult rat developed by Rivlin and Tator and described in section 5.3.1. This model simulates closely the clinical situation in which fragments of fractured vertebrae impinge temporarily and with forceful impact on the spinal cord, where they cause extensive mechanical damage. The model has been well established in our laboratory for several years.

We have previously shown in our rat model that administration of quercetin 1 hr after injury, continued in 12 hr-intervals promoted functional recovery in post-traumatically paralyzed hind limbs when treatment was continued for ten days (Schültke et al., 2003). The experiments herein reported were designed to answer the question whether shortening of treatment duration or delay of treatment onset would still result in functional recovery.

9.2 Materials and methods

A total of 105 adult male Wistar rats (9-10 weeks of age, 285 – 360g, Charles River Canada) were subjected to standardized mid-thoracic spinal cord injury. For all experiments, we used the model of spinal cord compression injury introduced by Rivlin and Tator (Rivlin and Tator, 1978), as described in section 5.3.1.

The most important criterion for our assessment of functional recovery after spinal injury with quercetin administration was somatic motor function. For this purpose, BBB and Angle board scores as described in section 5.4 were used throughout all experiments herein reported. Rating was made by two observers, of whom at least one was blinded to the treatment. The urinary bladders of all paraplegic animals were expressed manually three times daily until recovery of spontaneous voiding was achieved. The latter was observed in most animals, whether treated with quercetin or not, between weeks 2 and 3 after injury.

Animals were assigned to nine therapeutic groups or used as saline controls. Usually, surgery for two or three different experimental protocols was performed parallel, and to these two or three experimental groups, animals were assigned randomly. Also, for all groups containing more than seven animals, surgery was performed on half of the animals at two different time points at least eight weeks apart, so as to eliminate seasonal or other temporal variations as possibly related to differences seen. Surgery for all experiments described in this report was performed by the same researcher.

Therapy started at the earliest 1 hour after spinal cord compression injury, the minimum time in which clinical assessment of a patient with spinal cord injury could be reasonably expected. In one group (group 7), treatment onset was delayed to 12 hr after injury. In one group (group 8), treatment onset was as late as 2 weeks after injury. The

rational for this extremely late treatment onset was as follows: Scar formation, occurring relatively late in the process of wound healing, is driven at least partially by oxidative stress and inflammation. Since quercetin possesses both anti-oxidative and anti-inflammatory capacities (Huk, 1998; van Acker, 1995; Morel, 1993; Afanas'ev, 1988), we hypothesized that administration of the compound might be beneficial in this stage of the healing process. In most groups, animals received doses of 25 $\mu\text{mol/kg}$ per injection. This dose had been established in the earlier studies as optimal (Schültke et al., 2003). However, this dose appeared to become less effective with prolonged interval between injury and treatment onset, as seen in an experimental group for which treatment with 25 $\mu\text{mol/kg}$ quercetin was started at 12 hr after injury (group 7). Therefore, we decided to use a higher dose of quercetin for the delayed treatment onset experiments. After validating the first results of this late onset study with a second group of experimental animals, we introduced an additional group (group 9), to study whether the effects of early treatment onset and later stage high-dose therapy would potentiate each other.

Since studies have shown that the serum half-life of quercetin might be as long as 16 hr (Griffiths and Barrow, 1992), administration in 12 hr intervals appears reasonable. This assumption has been supported by the results of our first study, where 67% of animals recovered motor function sufficient to support walking after mid-thoracic spinal cord injury caused by an aneurysm clip with a calibrated closing force of 40 g (Schültke et al., 2003). However, we introduced two groups in which animals received quercetin three times daily (groups 4 and 6), in order to investigate whether those animals would show better recovery of motor function than their counterparts who received two daily doses of the compound.

Since our earlier studies had indicated that no statistically significant differences were seen between treatment duration of 4 and 10 days, several groups were introduced with shorter treatment duration, varying from one single injection to three days of treatment (groups 1 to 4). Three animals were submitted to laminectomy only, to exclude the possibility trauma other than spinal cord injury would impair motor function.

Summary and description of the various experimental protocols used are provided in Table 9.1.

	Start treatment after SCI	Duration of treatment	<i>Treatment schedule</i>	Total dose administered
Group 1 n = 6	1 hr	single injection	25 µmol/kg	25 µmol/kg
Group 2 n = 5	1 hr	24 hr	25 µmol/kg	75 µmol/kg
Group 3 n = 12	1 hr	72 hr	25 µmol/kg twice daily	175 µmol/kg
Group 4 n = 6	1 hr	72 hr	25 µmol/kg three times daily	250 µmol/kg
Group 5 n = 14	1 hr	10 days	25 µmol/kg twice daily	500 µmol/kg
Group 6 n = 5	1 hr	10 days	25 µmol/kg three times daily	750 µmol/kg
Group 7 n = 12	12 hr	10 days	25 µmol/kg twice daily	475 µmol/kg
Group 8 n = 15	2 weeks	3 weeks	75 µmol/kg twice daily	3.51 mmol/kg
Group 9 n = 7	1 hr + 2 weeks	10 days + 3 weeks	25 µmol/kg twice daily + 75 µmol/kg twice daily	4.01 mmol/kg
Group 10 n = 23	1 hr, 12 hr or 2 weeks	24 hr – 3 weeks	3 ml saline/ injection	no quercetin

TABLE 9.1: Summary of treatment protocols tested to establish the therapeutic window for quercetin administration.

n = animals in the group at beginning of the experiments

Seven animals of Group 5, six animals of Group 8, all animals of Group 9 and six saline controls were allowed to recover for 12 weeks after injury, to assess whether the improvement of motor function would be permanent beyond the end of the treatment period. All remaining animals were sacrificed six weeks after injury as described in section 5.5.

Statistical analysis: Parametric tests have been widely used to analyze for statistical significance of BBB scores. Given the qualitative structure of this scoring system, we feel that differences between any given two scoring levels, although seemingly equal when expressed in numbers, are in fact not really comparable with respect to quality. We therefore used a type of statistical analysis for binominal distribution (yes / no), with regard to the walking ability of the animals vs. their inability to walk, as more appropriate.

To perform this type of analysis, we used the following equation:

$$Sp = \sqrt{\frac{p \times (1 - p)}{N}}, \quad (1)$$

whereby Sp is the standard deviation of the sample p and n is the number in the sample (percentages or total numbers) (Pipkin, 1984). The 95% confidence interval is now multiplied by 1.96, i.e.:

$$x = Sp \times 1.96 \quad (2)$$

By subtracting the result of equation (2) to the percentage of walking animals we determined the lower value, and by adding the result of equation (2) we determined the upper value of the variance within 95% of the sample population:

$$\text{Low value: } p - x \quad (3a)$$

$$\text{High value: } p + x \quad (3b)$$

Statistical significance between data sets is generally assumed when $p < 0.05$, i.e. there is a less than 5% chance that data arise by chance alone. By defining the corner values for the variance within 95% of the population of a given data set, we determine that the data sets with a variance frame (defined by lower and upper values) which does not overlap with the variance frame of the first data set are statistically significantly different from the first data set.

9.3 Results

Of the 23 animals used as saline controls, one had to be euthanized because of excessive self-inflicted abdominal wounds (excessive gnawing) and one died during week two from unexplained causes. Two animals, one each from groups 5 and 7, were lost because of bladder rupture during manual expression.

None of the animals in any group was supporting weight when tested one week after injury. We had shown in previous experiments, that administration of 25 $\mu\text{mol/kg}$ quercetin, started 1 hr after injury and continued in 12 hr intervals for 10 days, was a very effective treatment schedule to support recovery of motor function in our animal model (Schültke et al., 2003). Therefore, we used this schedule (group 5) as pivotal point for the following experiments, which were designed to establish the therapeutic window for quercetin after acute traumatic spinal cord injury. With the ten-day treatment schedule, beginning 1 hr after spinal cord compression injury caused by a 5 sec closure of an aneurysm clip, calibrated to 50g closing force, around the mid-thoracic spinal cord, 46.2% (6 out of 13) animals recovered motor function sufficient to support walking (group 5). The plateau of recovery in this group was seen around four weeks after injury, more than two weeks after the end of the treatment cycle. No loss of function was observed in any of the animals, neither in those sacrificed at week 6, nor in those sacrificed at week 12 after injury (Table 9.2 and Fig. 9.1).

Time after injury	OUTCOME		
	BBB ≤ 8	BBB = 9	BBB ≥ 10
Week 1	1 / 2 / 8 / 1 / 8 / 3 / 8 / 8 / 5 / 2 / 0 / 5 / 4		
Week 2	6 / 1 / 6 / 6 / 5 / 1 / 8 / 5	9 / 9 / 9	10 / 10
Week 3	8 / 5 / 7 / 8 / 5 / 5 / 5	9	11 / 11 / 13 / 12 / 10
Week 4	7 / 6 / 8 / 8 / 5 / 6 / 7		12 / 12 / 14 / 12 / 10 / 10
Week 6	8 / 6 / 8 / 7 / 7 / 7	9	12 / 12 / 14 / 14 / 11 / 12
Week 12	8 / 6 / 8	9	13 / 15 / 16

Table 9.2: Group 5, BBB score distribution through weeks 1 –12.

Treatment with 25 µmol/ kg quercetin started 1 hr after injury,
and continued for 10 days in 12 hr intervals.

Six out of 13 animals (46.2%) recovered sufficient hind limb function to walk.

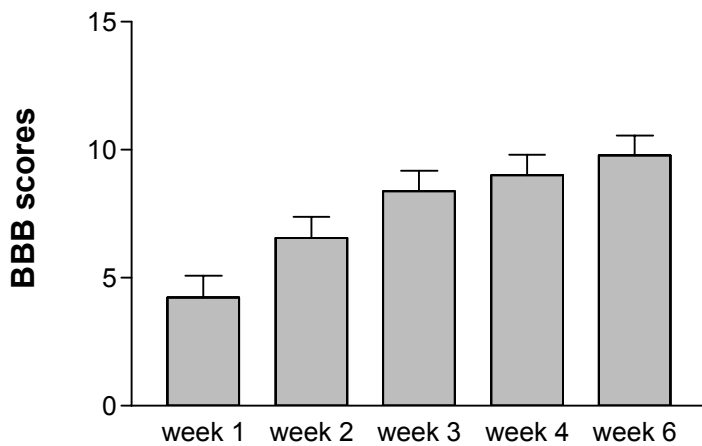


Figure 9.1: BBB scores for recovery of animals in group 5 during the six-week observation period. Means \pm SD.

Treatment with 25 $\mu\text{mol/ kg}$ quercetin started 1 hr after injury, and continued for 10 days in 12 hr intervals.

Changing the treatment schedule to three instead of two daily injections did not improve recovery of function. On the contrary, none of the animals injected three times daily was able to walk at the end of the six week recovery period (group 6). At the end of the observation period, three of the five animals (60%) were supporting weight in stance (Table 9.3).

Time after injury	OUTCOME		
	BBB ≤ 8	BBB = 9	BBB ≥ 10
Week 1	5 / 1 / 2 / 5 / 2		
Week 2	7 / 3 / 3 / 6 / 6		
Week 3	8 / 1 / 8 / 8	9	
Week 4	6 / 5	9 / 9 / 9	
Week 6	8 / 6	9 / 9 / 9	

Table 9 .3: Group 6, BBB score distribution through weeks 1 – 6.

Treatment with 25 μ mol/kg quercetin started 1 hr after injury, and continued for 10 days in 8 hr intervals.

None of the animals recovered sufficient hind limb function to walk.

Shortening the treatment time, on the other hand, appears to be rather an advantage. For animals in group 3, duration of treatment was reduced to 3 days. Seven out of 12 animals (58%) recovered sufficient motor function to support walking, and one animal supported weight in stance. Compared to the animals receiving the ten-day treatment cycle under otherwise identical conditions (group 5), animals in group 3 reached a good level of performance (recovery of motor function) in the same interval (two to three weeks) after injury (Table 9.4 and Fig. 9.2). The maximum levels of recovery appeared slightly lower in the group which received treatment for only three days (BBB 10-12, as compared to BBB 10-14 for ten days treatment). More animals recovered motor strength in their hind limbs

sufficient to walk with three day treatment duration, compared to 10 days treatment duration on an otherwise identical treatment schedule. This was statistically significant (Fig. 9.3)

Time after injury	OUTCOME		
	BBB ≤ 8	BBB = 9	BBB ≥ 10
Week 1	8 / 1 / 1 / 1 / 6 / 7 / 1 / 1 / 6 / 0 / 1 / 8		
Week 2	2 / 8 / 7 / 2 / 1 / 0 / 8	9	10 / 11 / 10 / 10
Week 3	2 / 8 / 8 / 2 / 2 / 1	9	10 / 10 / 11 / 10 / 11
Week 4	3 / 6 / 5 / 1		11 / 10 / 10 / 11 / 12 / 12 / 10 / 10
Week 6	2 / 5 / 6 / 3	9	11 / 10 / 10 / 12 / 10 / 10 / 11

Table 9.4: Group 3, BBB score distribution through weeks 1 – 6.

Treatment with 25 µmol/kg quercetin started 1 hr after injury, and continued for 3 days in 12 hr intervals.

Seven out of 12 animals (58%) recovered sufficient hind limb function to support stepping / walking.

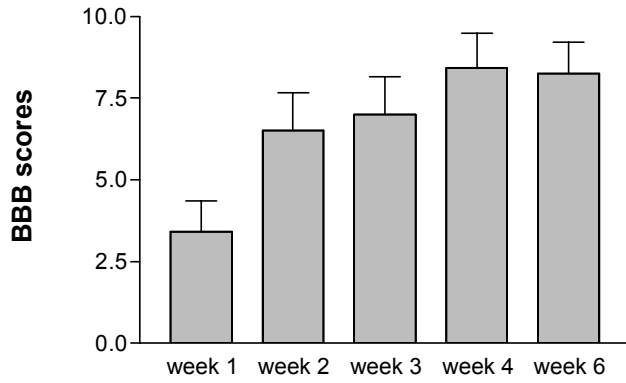


Figure 9.2: Group 3, BBB score distribution through weeks 1 – 6. Treatment with 25 $\mu\text{mol/kg}$ quercetin started 1 hr after injury, and continued for 3 days in 12 hr intervals. Means \pm SD.

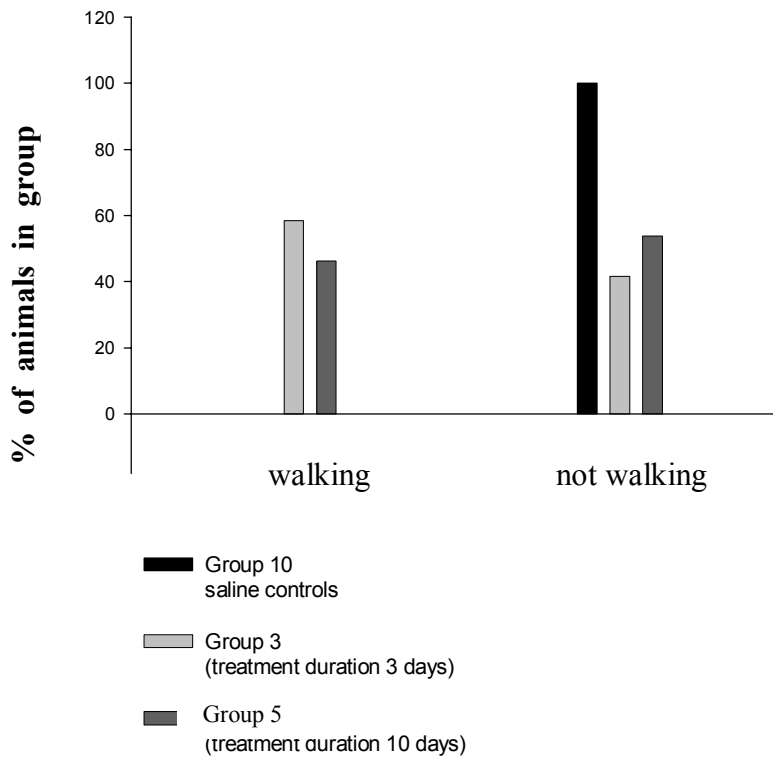


FIGURE 9.3: Outcomes 3 days treatment vs. 10 days treatment duration with 25 $\mu\text{mol/kg}$ quercetin in 12 hr intervals; walking vs non-walking animals, at six weeks after injury.

Animals in group 4 received treatment according to an identical schedule as animals in group 3, with the exception that 25 $\mu\text{mol/kg}$ quercetin were administered three times, instead of two times, daily. None of the animals recovered sufficient hind limb function to walk or support weight in stance (Table 9.5).

Time after injury	OUTCOME		
	BBB \leq 8	BBB = 9	BBB \geq 10
Week 1	1 / 8 / 2 / 1 / 0 / 2		
Week 2	6 / 7 / 2 / 2 / 1 / 5		
Week 3	6 / 6 / 5 / 2 / 1 / 6		
Week 4	6 / 5 / 2 / 6 / 2 / 8		
Week 6	7 / 6 / 3 / 6 / 3 / 8		

Table 9.5: Group 4, BBB score distribution through weeks 1 – 6.

Treatment with 25 $\mu\text{mol/kg}$ quercetin started 1 hr after injury, and continued for 3 days in 8 hr intervals.

None of the animals recovered sufficient hind limb function to walk.

Increasing the frequency of quercetin administration from twice to three times daily in an otherwise unchanged treatment protocol resulted in loss of its beneficial effect on recovery of motor function, as seen in the results from both groups 4 and 6. None of the

animals receiving 25 $\mu\text{mol/kg}$ quercetin thrice daily regained sufficient hind limb function to walk.

Shortening of treatment duration to less than 3 days resulted in significantly decreased of therapeutic benefit (Table 9.6; Fig. 9.4). With a treatment duration of 24 hr (3 injections; Group 2), one animal out of 5 was stepping (BBB 10), none was walking and none was weight supporting in stance with 24 hr treatment duration (group 2). Out of five animals in group 1 (single injection), one animal out of five was stepping and one animal was walking at 6 weeks after injury. With the results of only 33.3% (group 1) or 20% (group 2) animals stepping or walking, both treatment schedules were therefore judged unsatisfactory.

Time after injury		OUTCOME		
		BBB \leq 8	BBB = 9	BBB \geq 10
Week 1	Group 1	1 / 2 / 0 / 1 / 1 / 1		
	Group 2	2 / 2 / 0 / 0 / 1		
Week 2	Group 1	1 / 5 / 1 / 8 / 1		10
	Group 2	2 / 1 / 1 / 6 / 7		
Week 6	Group 1	3 / 6 / 1 / 5		11 / 10
	Group 2	6 / 7 / 1 / 6		10

Table 9.6: Groups 1 and 2, BBB score distribution through weeks 1 – 6.

Treatment with 25 $\mu\text{mol/kg}$ quercetin started 1 hr after injury,

One single injection (group 1) or 24 hr treatment duration (group 2).

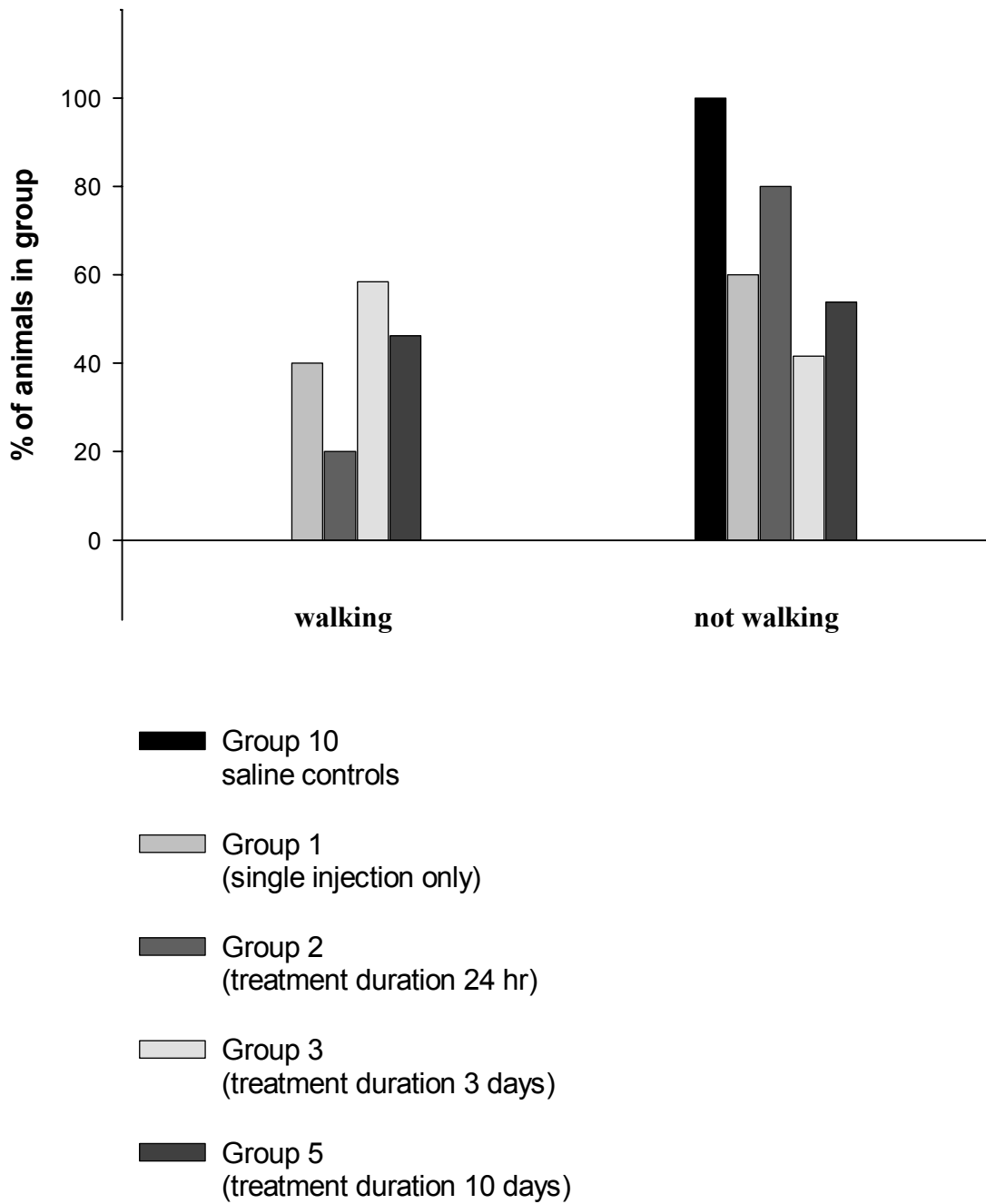


FIGURE 9.4: Outcomes, all groups beginning treatment 1hr after injury, single injection or continued in 12 hr intervals vs. saline controls, walking vs. non-walking animals at 6 weeks after injury.

Next, we introduced one group in which animals received 25 $\mu\text{mol/kg}$ quercetin for the duration of 10 days, yet treatment was started only 12 hr after injury (group 7). Out of 11 animals, only two (18.2%) regained sufficient hind limb function to support walking, and one animal supported weight in stance (Table 9.7).

Time after injury	OUTCOME		
	BBB \leq 8	BBB = 9	BBB \geq 10
Week 1	3 / 0 / 1 / 1 / 1 / 5 / 8 / 7 / 5 / 8 / 1		
Week 2	2 / 1 / 2 / 2 / 6 / 5 / 5 / 2 / 7 / 2	9	
Week 3	6 / 1 / 1 / 6 / 8 / 6 / 2 / 5 / 6	9	11
Week 4	6 / 1 / 1 / 6 / 8 / 6 / 7 / 6	9	11 / 12
Week 6	7 / 1 / 2 / 6 / 8 / 4 / 6 / 5	9	12 / 12

Table 9.7: Group 7, BBB score distribution through weeks 1 – 6.

Treatment with 25 $\mu\text{mol/kg}$ quercetin started 12 hr after injury, and continued for 10 days in 12 hr intervals.

Speculating that a higher dose of quercetin might be beneficial when administered late after injury, we introduced one group that received 75 $\mu\text{mol/kg}$ twice daily for 3 weeks duration. Treatment onset was 2 weeks after injury. Surprisingly, a significant rate of recovery was indeed seen in this group (group 8). To validate those results, this experimental protocol was repeated twice, each time yielding a similar distribution of BBB scores throughout the subgroup. None of the animals in this group were stepping / walking or supported weight in stance at the time of treatment onset (day 15 after injury). Recovery of motor function sufficient to support walking was seen in 40% (6 out of 15) animals in this group. In those animals, which recovered with the delayed treatment onset, slight improvements were seen already one week after treatment onset. Recovery usually reached a plateau at week 5 after injury, which coincided with the end of the treatment period. In none of the animals in this group, six of which were allowed to recover until the end of week 12 after injury, was any deterioration of function seen after the plateau of recovery had been reached (Table 9.8 and Figure 9.5).

Time after injury	OUTCOME		
	BBB ≤ 8	BBB = 9	BBB ≥ 10
Week 1	8 / 1 / 2 / 2 / 1 / 1 / 2 / 1 / 8 / 2 / 0 / 1 / 3 / 2 / 8		
Week 2	8 / 8 / 8 / 2 / 1 / 8 / 3 / 8 / 2 / 2 / 3 / 7 / 6 / 4 / 8		
Week 3	8 / 1 / 6 / 6 / 8 / 8 / 6	9 / 9 / 9 / 9 / 9 / 9	11 / 10
Week 4	5 / 8 / 8 / 8 / 7 / 6 / 8	9 / 9 / 9	11 / 11 / 12 / 10 / 10
Week 5	6 / 8 / 8 / 6 / 8 / 8 / 5	9 / 9	11 / 10 / 12 / 11 / 12 / 11
Week 6	8 / 8 / 8 / 5 / 8 / 8 / 8	9 / 9	11 / 11 / 12 / 12 / 11 / 10
Week 12 (subset of animals tested at 6 weeks after injury)	8 / 7 / 8		11 / 10 / 12

Table 9.8: Group 8, BBB score distribution through weeks 1 – 12.

Treatment with 75 $\mu\text{mol/kg}$ quercetin was started 2 weeks after injury, and continued in 12 hr intervals for 3 weeks.

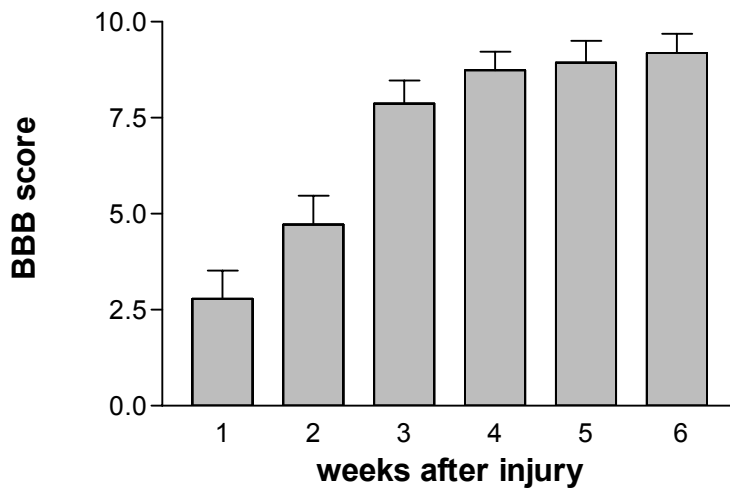


Figure 9.5: BBB scores of animals with delayed treatment (group 8) during the 6 week observation period. Means \pm SD.

The combination of a ten-day treatment cycle, beginning at 1 hr after injury, with high-dose treatment during weeks 3-5 did not show the expected effect of potentiation in recovery potential (group 9) (Table 9.9 and Fig. 9.6). Rather, with 42.8% (3 out of 7) animals, the percentage of animals recovering motor function sufficient to support walking was similar to that expected for the 10-day protocol alone (group 5).

Time after injury	OUTCOME		
	BBB ≤ 8	BBB = 9	BBB ≥ 10
Week 1	8 / 8 / 2 / 8 / 2 / 8 / 1		
Week 2	8 / 6 / 7 / 7	9 / 9 / 9	
Week 3	8 / 8 / 8 / 7	9 / 9	10
Week 4	8 / 8 / 8 / 8	9	11 / 11
Week 6	8 / 8 / 8 / 7		11 / 11 / 10

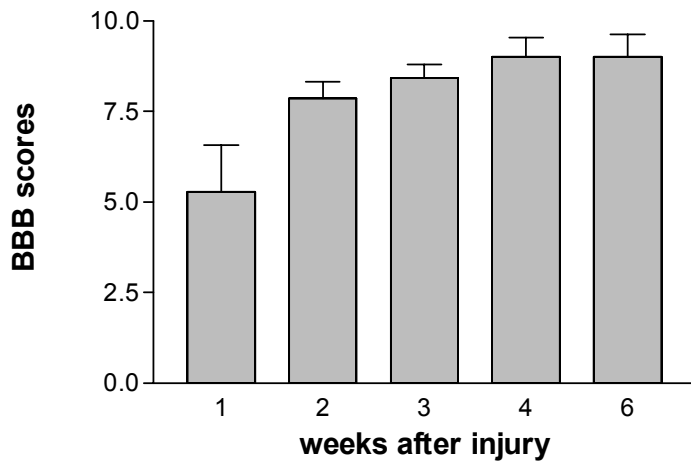


Figure 9.6

Table 9.9 and Figure 9.6: Group 9, BBB score distribution through weeks 1 – 6.

Treatment with 25 $\mu\text{mol/kg}$ quercetin started 1 hr after injury, 10 days duration, followed by 3 weeks treatment with 75 $\mu\text{mol/kg}$ quercetin twice daily, starting 2 weeks after injury.

Within the group of animals receiving saline vehicle only, none of the animals recovered sufficient motor function to support walking or stepping movements at any time, and only one out of the 21 control animals (4.8%) was able to support weight in stance (Table 9.10). No differences in outcome distribution were observed with regard to the time of onset or duration of saline administration.

None of the three animals, which underwent laminectomy only (no spinal cord injury), showed any neurological impairment. They all received BBB scores of 21 at every testing session.

Time after injury	BBB ≤ 8	BBB 9
Week 1	3 / 7 / 1 / 0 / 0 / 2 / 1 / 1 / 0 / 1 / 1 / 6 / 1 / 1 / 5 / 6 / 1 / 8 / 2 / 1 / 2	
Week 2	3 / 6 / 2 / 2 / 2 / 2 / 2 / 6 / 1 / 2 / 2 / 1 / 1 / 1 / 8 / 3 / 1 / 8 / 2 / 1 / 2	
Week 6	8 / 8 / 6 / 8 / 7 / 8 / 8 / 1 / 1 / 2 / 6 / 1 / 7 / 8 / 6 / 1 / 2 / 7 / 3 / 6	9
Week 12	7 / 6 / 7 / 8 / 7 / 1	9

Table 9.10: Group 10 (saline injections), BBB scores through weeks 1 –12.

Recovery of motor function for the animals in the various treatment protocols has been summarized in Table 9.11.

	Start treatment after SCI	Duration of treatment	Treatment schedule	Outcome		
				≤ 8	9	≥ 10
Group 1 n = 6	1 hr	single injection	25 µmol/kg	4	0	2
Group 2 n = 5	1 hr	24 hr	25 µmol/kg	4	0	1
Group 3 n = 12	1 hr	72 hr	25 mol/kg twice daily	4	1	7
Group 4 n = 6	1 hr	72 hr	25 µmol/kg three times daily	6	0	0
Group 5 n = 13	1 hr	10 days	25 µmol/kg twice daily	6	1	6
Group 6 n = 5	1 hr	10 days	25 µmol/kg three times daily	2	3	0
Group 7 n = 11	12 hr	10 days	25 µmol/kg twice daily	8	1	2
Group 8 n = 15	2 weeks	3 weeks	75 µmol/kg twice daily	7	2	6
Group 9 n = 7	1 hr + 2 weeks	10 days + 3 weeks	25 µmol/kg twice daily + 75 µmol/kg twice daily	4	0	3
Group 10 n = 21	1 hr, 12 hr or 2 weeks	24 hr – 3 weeks	3 ml / injection	20	1	0

TABLE 9.11: Summary of outcomes with the various treatment schedules.
n = surviving animals at end of experiment.
BBB ≤ 8: no weight support, BBB 9: weight support in stance,
BBB ≥ 10: stepping / walking

	Start treatment after SCI	Duration of treatment	<i>Treatment schedule</i>	Animals walking	Variance and statistical significance from group 5 (*)
Group 1 n = 6	1 hr	single injection	25 µmol/kg	33.3 %	*
Group 2 n = 5	1 hr	24 hr	25 µmol/kg	20 %	*
Group 3 n = 12	1 hr	72 hr	25 µmol/kg twice daily	58 %	* significantly better
Group 4 n = 6	1 hr	72 hr	25 µmol/kg three times daily	none	*
Group 5 n = 14	1 hr	10 days	25 µmol/kg twice daily	46.2 %	35.6 – 56.8%
Group 6 n = 5	1 hr	10 days	25 µmol/kg three times daily	none	*
Group 7 n = 12	12 hr	10 days	25 µmol/kg twice daily	18.2 %	*
Group 8 n = 15	2 weeks	3 weeks	75 µmol/kg twice daily	40 %	
Group 9 n = 7	1 hr + 2 weeks	10 days + 3 weeks	25 µmol/kg twice daily + 75 µmol/kg twice daily	42.8 %	
Group 10 n = 23	1 hr, 12 hr or 2 weeks	24 hr – 3 weeks	3 ml saline/ injection	none	*

Table 9.12:

Statistical analysis for binominal distribution.

Outcome for delayed treatment (groups 8 and 9) is not statistically significant from that in Group 5 (i.e. early treatment), while the difference in outcome of all other schedules is statistically significant.

Asterisk: outcome statistically significant, compared to Group 5

Correlation of BBB and Angle board scores

For this comparison, test results of 107 animals were analyzed. All animals received both BBB scores and Angle board scores on a weekly basis. The following correlation was found between BBB and Angle board scores (Tables 9.12 and 9.13):

BBB scores (points)	Angle board scores (angular degrees)
Healthy animals (BBB 21)	45-50
Animals walking, but incompletely recovered, coordination of for and hind limb movements (BBB ≥ 12)	45 - 50
Animals walking, but incompletely recovered, no coordination of for and hind limb movements (BBB 10 - 11)	35 – 45
Animals supporting weight, but not walking (BBB 9)	30 - 40
Animals not supporting weight, with strong movements in all three joints of the hind limbs (BBB 7 or 8)	25 - 35
Animals not supporting weight, (BBB 2 - 6)	25 - 30
No movement or weak movement of one or two joints (BBB 0 or 1)	< 25

TABLE 9.13: Correlation between functionally distinct groups of animals, which are also distinct by BBB scores, and Angle board scores.

Angle board scores	BBB scores
< 25	0 - 1
25	1 - 8
30	1 - 9
35	1 - 11
40	5 - 11
45	8 - 21
50	11 - 21

TABLE 9.14: Numeral correlation of BBB scores to Angle board scores.

9. 4 Discussion

In a recent review of pathophysiology and treatment of spinal cord injury, Hulsebosch (2002) points out a list of possible targets for intervention in patients with acute traumatic spinal cord injury. The first three items on this list refer to reduction of edema and free radical production, rescue of neural tissue at risk of dying in secondary processes and control of inflammation. In an earlier review, Juurlink and Paterson as well had suggested that the non-transected axons spared after acute spinal cord injury might be a good target for therapeutic intervention (Juurlink and Paterson, 1998). In both compression and contusion type injuries, the involvement of various pathological mechanisms has been implied in secondary loss of those axons spared by the primary injury, including demyelination caused by apoptosis of oligodendrocytes and inflammatory processes (Gledhill et al., 1973; Harrison and McDonald, 1977; Griffiths and McCulloch, 1983; Blight, 1985; Shuman et al., 1997). Tissue damage caused by inflammatory processes is mediated by neutrophils and macrophages invading the site of injury and its immediate surroundings. Neutrophils and macrophages secrete oxidants as well as hydrolytic enzymes into the extracellular space, where they react with both damaged and healthy tissue (Pincemail et al., 1988; Rodrigues et al, 2002). Since quercetin had been shown to possess anti-edematous, anti-oxidant and anti-inflammatory capacities, it was reasonable to expect this compound to act neuroprotective in the setting of acute traumatic spinal cord injury. The results of our experiments indicate that this assumption is correct.

In our earlier experiments, in which the calibrated closing force of the aneurysm clip was 40 g, 67% of initially paraplegic animals recover sufficient hind limb function to walk (Schültke et al., 2003). To create the spinal cord injury for the experiments in group 5, an

identical experimental protocol was used, with the one exception that the calibrated closing force of the aneurysm clip was 50 g, instead of 40 g. In these experiments, recovery of hind limb function sufficient to support walking was seen only in 46.2% of the animals. This suggests that the efficacy of quercetin after acute traumatic spinal cord injury is limited by the severity of the trauma. The average of BBB scores within the group of stepping / walking animals after 40 g clip injury was 14.6 vs. 13.7 in the group submitted to 50 g clip injury. Keeping in mind that the inter-observer difference for the BBB scoring system is two points, this difference is not statistically significant, although it might indicate a tendency towards better recovery of coordination between fore and hind limbs in animals with a less severe injury.

Increasing administration of quercetin to three times daily, virtually eliminated the beneficial effect on recovery of motor function that had been seen with twice daily administration in otherwise identical treatment protocols (groups 5 vs. 6 and groups 3 vs. 4). Even with thrice daily administration (75 $\mu\text{mol/kg}$ quercetin / day), daily doses administered in the early phase after injury are only half that administered in the late-onset experiments (group 8). Yet, not a single animal recovered to BBB > 8 in these groups, while a significant percentage of animals were walking in both groups with late high-dose treatment. While the higher doses of quercetin have a beneficial effect on recovery of motor function when administered in the later phase, no benefit is seen with administration early after injury. This suggests that quercetin levels might play a significant role in determining treatment outcome early after injury, but not so much in a later phase. Although we have not measured quercetin levels in either blood or tissue of our animals, the idea is supported by the results obtained from our experiments. Comparing results within the two groups receiving late high-

dose treatment (groups 8 and 9), it is noteworthy that the percentage of walking animals in both groups was about equal (40 vs. 43%). This furthermore indicates that the higher dose administered in a later phase after injury did not result in any loss of recovery gained with treatment early after injury.

Although it appears to be established that the activity of neutrophils as cellular mediator of inflammation causes tissue damage, the role of macrophages in the injury process is much less straightforward. It seems that activity of macrophages and microglia play a rather important role in the repair processes after tissue injury (Schwartz et al., 1999; Rapalino et al., 1998; Prewitt, 1997). Myeloperoxidase is stored in the granules of neutrophils. Reacting with hydrogen peroxide and chloride anions, hypochlorous acid and chloramines are formed (Pincemail et al., 1988). When hypochlorous acid and chloramines are released into the extracellular space, they will cause excessive tissue damage to protein structures (Hawkins and Davies, 1998). One of the mechanisms, through which quercetin acts, is the inhibition of myeloperoxidase activity. It therefore appears reasonable to inhibit the myeloperoxidase activity of neutrophils by administration of quercetin. Alternatively, decreased myeloperoxidase activity might be caused by reduced influx of neutrophils at the site of injury, which might be due to administration of quercetin. As Carlson and colleagues have described, myeloperoxidase activity caused by neutrophil invasion peaks around 24 hr after spinal cord injury in the rat, declining within 48 hr (Carlson et al., 1998). Extending treatment duration beyond the phase of high myeloperoxidase activity might lead to a situation in which quercetin interferes with signaling pathways necessary to attract macrophages, leading to a disturbance of repair functions usually performed by those cells. It, therefore, might not be advisable to abrogate macrophage function completely.

Phagocytosis of debris from the injury site is one of the important functions of macrophages in the complex of repair processes. Fadok and colleagues have shown in an in-vitro model that macrophages, which have ingested apoptotic cells, contribute to decrease in production of pro-inflammatory cytokines, including TGF β (Fadok et al., 1998). It also has been suggested that macrophages might take up myeloperoxidase from the injured tissue, either by phagocytosis of myeloperoxidase-containing neutrophils or by another direct process, thereby decreasing the potential of secondary injury (Winterbourn et al., 2000; Hampton et al., 2002; Rodrigues et al., 2002). Interestingly, it has been shown in an in-vitro model of phagocytotic macrophage activity, that quercetin decreased the amount of myelin phagocytosed by stimulated macrophages (Hendriks et al., 2003). If this were true also under in-vivo conditions, quercetin might act neuroprotective by preserving the intactness of myelin sheaths.

To explain our observation that administration of quercetin as late as 2 weeks after injury still results in improved recovery of motor function, two prominent pathomechanisms, active late after injury, come to mind: apoptosis and glial scar formation. It has been shown in rat as well as in monkey models that both neurons and glia can die by apoptosis (Beattie et al., 1992; Crowe et al., 1997; Liu et al., 1997). Apoptotic cell death is expected to contribute to the increasing volume of secondary injury, probably weeks to even months after injury (Johnson et al., 1995). In fact, it has been shown in vitro that quercetin is an excellent inhibitor of apoptotic nuclear damage (Choi et al., 2003).

The other process possibly influenced by late administration of quercetin is glial scar formation. In autopsy studies of human spinal cord injury, gliosis and macrophage infiltration around a central necrosis at the site of injury were found as late as 5 months after acute

traumatic spinal cord injury (Ito et al., 1997). It has been shown that the expression of TGF β 2 is elevated in activated macrophages and glia after spinal cord injury. While oligodendrocytes in the uninjured spinal cord are negative for TGF β 2, TGF β 2 expression increases through day 10 after spinal cord injury, plateaus and then decreases again, having almost disappeared by day 30 after SCI (Lagord et al., 2002). Secretion of TGF β 2 promotes secretion of extracellular matrix molecules, and therefore scar formation (Lagord et al., 2002; Kovacs et al, 1991). We have not found any direct evidence for interaction of quercetin and TGF β 2 in the literature. However, evidence has been found, that quercetin reduces the extent of hypertrophic scar formation by inhibition of fibroblast proliferation (Phan et al., 2003). Since TGF β 2 promotes fibroblast proliferation, TGF β 2 might well be the pathway through which quercetin affects fibroblast proliferation. This idea is supported by the reduction of gliosis (the equivalent of scar formation in CNS tissue) and glial fibrillary acidic protein (GFAP) levels by quercetin seen in a scratch injury model of primary astrocyte cultures (Wu and Yu, 2000).

The correlation of BBB scores and Angle board scores in our experiments, derived from more than 650 individual test situations, clearly shows that the BBB scoring system is much more sensitive and clinically more relevant for the assessment of motor function recovery (Tables 9.12 and 9.13). While the BBB system allows a clear distinction between healthy animals, animals incompletely recovered but walking with coordination of limb movements, and animals stepping / walking without coordination of limb movements, no such distinction is possible when the Angle board scores are used. In the lower range of motor function recovery (BBB \leq 9), again overlap of Angle board scores from three functionally distinct groups is encountered (Table 9.12). It is noteworthy that identical Angle

board scores have been obtained for animals with clinically significant different levels of hind limb motor function (Table 9.13). The same Angle board score of 35 could have been assigned to an animal with only weak movements in one or two joints, or to an animal which has recovered sufficient hind limb function to support stepping / walking.

Our experiments have shown that there is a therapeutic window of two weeks duration for onset of quercetin treatment, when the dose is adjusted. Quercetin might act neuroprotective by attenuating similar pathomechanisms of secondary tissue injury in the early and late phases after injury, such as decrease of activated caspase-3 expression. Although the pathways through which quercetin acts neuroprotective still requires further investigation, the results of our studies strongly suggest that administration of quercetin after acute spinal cord injury is neuroprotective.

9.5 References

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Chapter 10

Discussion and Conclusions

10.1 Summary of results

The results of our experiments have clearly established that

- 1) Administration of quercetin, starting one hour after trauma and continued in 12 hr intervals, promotes recovery of motor function in an animal model of acute spinal cord compression injury. Contrary to our expectation, that quercetin would be neuroprotective only when administered early after injury, we found that the compound contributed significantly to recovery of motor function even when administered as late as 2 weeks after trauma.
- 2) Administration of quercetin, starting one hour after trauma and continued in 12 hr intervals, results in preservation of compound action potentials in an animal model of moderate fluid percussion injury, a model for human brain concussion.
- 3) Quercetin conveys neuroprotection through interference with several mechanisms which otherwise would contribute to the increase of post-traumatic lesion volume after acute traumatic spinal cord injury. Those mechanisms include decrease of the myeloperoxidase activity caused by neutrophils invading the site of injury, inhibition of apoptotic pathways converging on caspase-3, and chelation of free iron ions.

10.2 The first hypothesis is upheld

We hypothesized that the ability of the polyphenolic flavonoid quercetin to chelate ferrous iron (Fe^{+2}) and to decrease the intensity of post-traumatic inflammatory processes will result in improved recovery of motor function after neurotrauma.

The strategy for our first block of experiments, as described in chapter 6, was based on the assumption that iron chelation would be an important component in the neuroprotective action profile of quercetin. The MRI-based chelation experiments allowed us to determine in a relatively short time and at little expense a quercetin dose well suited to reduce the availability of free iron ions *in vitro*. Had the MRI not been available, a substantial number of animals would have been required to perform those experiments in the animal model. Our first animal experiments were designed as a dose-response study, to explore the relationship of administered quercetin dose and therapeutic effect as reflected in improved recovery of motor function. We were able to demonstrate that intraperitoneal administration of 25 $\mu\text{mol} / \text{kg}$ quercetin, if treatment was started 1 hr after injury and continued in 12 hr intervals, promoted significant recovery of motor function in the previously paraplegic hind limbs of our animals, when compared to animals receiving saline vehicle only. Thus, we found that the quercetin dose resulting in optimal iron chelation in the MRI experiments also resulted in significantly improved recovery of motor function in the setting of spinal cord injury. This supports the hypothesis that iron chelation is indeed an important component in the neuroprotective action profile of quercetin. Post-traumatic hemorrhages are a frequent occurrence after acute spinal cord trauma. Disintegrating erythrocytes are the source for hemoglobin, which passes through several stages of disintegration from oxyhemoglobin via deoxyhemoglobin to methemoglobin, before cells break down (Bradley, 1993).

Hemosiderin, a breakdown product of hemoglobin, tends to remain at the site of injury, awaiting further breakdown of the molecule. Serum iron, ferritin and transferrin levels are indirect indicators of an organism's overall iron load. However, those parameters will fail to give us any information on the availability of iron-containing breakdown products concentrated at the site of injury. Therefore, no valuable information can be derived from them regarding the oxidative stress potential caused by the locally confined presence of iron. On the other hand, it has been shown that the presence of iron causes signal changes in both T1 and T2 weighed MRI images. Hemosiderosis has been shown to significantly decrease T2 relaxation time in a dose-dependent fashion (Salo et al., 2002). During the first two or three days after trauma, the cellular integrity of erythrocytes stays mostly intact and mainly hemoglobin and deoxyhemoglobin are present. Although deoxyhemoglobin molecules create a stronger magnetic field than deoxyhemoglobin, only the duration of the T2 signal is shortened as a consequence, while the T1 signal (with shorter acquisition times than T2 signals) is almost unaffected. This can be explained by the fact that heme iron in both oxyhemoglobin and deoxyhemoglobin is in the ferrous state (Fe^{2+}), with four unpaired electrons. However, after about 3 days, oxidative disintegration of hemoglobin has advanced to form methemoglobin, which is at first present intracellularly, but found increasingly extracellularly with progressive disintegration of the erythrocytes (Wintrobe et al, 1981). Iron in methemoglobin is bound in the ferric state (Fe^{3+}), with five unpaired electrons. The paramagnetic strength of molecules is based on the number of unpaired electrons in a molecule (Bloemberger et al., 1948). Since the methemoglobin molecule has five unpaired electrons, as compared to the four unpaired electrons found in deoxyhemoglobin, the magnetic field created is stronger in methemoglobin. Water molecules passing through this

magnetic field will experience stronger dephasing due to dipole-dipole interaction, which is reflected in shortening of the T1 signal (Bradley; 1993). We modeled chelation of iron ions in both the ferrous and ferric states by the flavonoid quercetin. Since changes in the behavior of the T1 signal appear more continuous than T2 changes, where differences between ferrous and ferric states of iron are concerned, we chose analysis of T1 signal changes for our MRI experiments. Methemoglobin (containing ferric iron), hydrogen peroxide and superoxide radicals are produced during the disintegration process of hemoglobin by autooxidation (Winterbourn, 1985). All of those disintegration products are known to be involved in oxidant processes contributing to lipid membrane peroxidation (Juurlink and Paterson, 1998). Chelation of both ferrous and ferric iron ions by quercetin has been demonstrated in our MRI experiments and in experiments performed elsewhere, but it was more significant for ferrous iron. Since MRI was not available for us to image the spinal cords of our animals in the post-traumatic state, we reverted to indirect assessment of iron in the spinal cord at the injury site. At four weeks after injury, all hemoglobin could be expected to have disintegrated to a state where no oxyhemoglobin or deoxyhemoglobin (i.e. no ferrous iron-containing heme-molecules, originating from post-traumatic hemorrhage) were present any more. Ferric iron can be easily detected using a Mallory iron stain on tissue sections. In our spinal cord injury experiments described in chapter 7, no ferric iron was detected by Mallory iron stain in spinal cord tissues of injured animals at 4 weeks after the trauma when quercetin had been administered, contrary to animals which received saline vehicle only, where ferric iron was present. This suggests that, in our in-vivo experiments, the administered quercetin had chelated ferrous iron and thereby prevented its conversion to ferric iron. Thus, we have

shown that quercetin facilitates iron clearance in the setting of acute traumatic spinal cord injury.

10.3 Neuroprotection by quercetin: antioxidant, anti-inflammatory and anti-apoptotic

In experiments performed elsewhere, quercetin had been shown to have antioxidant and anti-inflammatory actions both *in vitro* and *in vivo*. However, no experiments had been performed to test the action of quercetin in the settings of CNS trauma. Since, from the compound's known action profile, a neuroprotective effect could be expected, we investigated whether this neuroprotective effect would be reflected in decreased activity of the pro-inflammatory enzyme myeloperoxidase and whether the extent of apoptotic cell death could be decreased by quercetin administration. When we found, in our short-term experiments, that both myeloperoxidase activity and indicators for apoptotic cell death were down-regulated by administration of quercetin, we designed a new block of long-term experiment that would allow us to assess whether the compound's neuroprotective effect seen with biochemical and immunocytochemical tissue analysis would finally translate into preservation of motor function. Since spinal cord trauma is frequently associated with head trauma, we used animal models for both presentations of CNS trauma for our experiments. Although different strains of rats were used for spinal cord trauma and head trauma experiments, animals in both experimental settings were equal in species and gender. Minor differences existed in age and weight of the animals. It has been well documented that cycling hormones like estrogen and progesterone significantly modulate an organism's response to CNS trauma (Galani et al., 2001; Shugrue and Merchenthaler, 2003; Sribnick et al., 2003). Administration of progesterone has been shown to attenuate cerebral edema after

traumatic brain injury (Shear et al., 2002) and supported sparing of neurons from secondary injury, which resulted in improved cognitive outcome (Roof et al., 1997). Therefore, we used exclusively male animals for our experiments, so as to avoid possible interference with hormonal modulators of post-traumatic preservation and repair processes. Our choice of experimental subjects would assure that statements about potential neuroprotection made based on the results of those experiments were as closely related as if two different sets of data were collected in a population of male human patients for head injury and spinal cord trauma.

It should be noted that myeloperoxidase activity was diminished in both spinal cord injury and head trauma after administration of the same dose of quercetin. This is interesting because it further supports the idea of similarities between pathomechanisms involved in different forms of CNS injury. The fact is also important with regard to possible clinical trials. Since the same dose of a compound is shown to have beneficial effects in both types of injury, which was proven on both functional and biochemical level, we have excluded a potential problem that might have arisen if the compound were beneficial in one but harmful in the other setting. Having excluded this potential problem, we would not have to restrict the indication for our compound to cases of isolated spinal cord injury.

Hydrogen peroxide apparently plays a major role for several pathways leading to secondary CNS injury. Released by stimulated neutrophils, hydrogen peroxide contributes to activation of apoptotic pathways converging on caspase-3 (Lu et al., 2001). Casha and colleagues (2001) have shown in adult rats that, at the site of injury, caspase-3 was completely cleaved (i. e. activated) between days 1 and 7 post trauma and associated with extensive glial apoptosis at the site of injury. Although in Casha's study, only glial apoptosis

was reported, results from our own study indicate that apoptotic cell death after SCI affects both glia and neurons. Our findings agree with those reported by Liu and colleagues (1997). We found that in traumatized spinal cord tissue caspase-3 was significantly activated at three days after injury, when only saline vehicle had been administered. However, with administration of 25 $\mu\text{mol/kg}$ quercetin, starting one hour after injury and continued in 12 hr-intervals, caspase-3 activation was significantly decreased. Caspase-3 activation can be induced by a variety of trauma-associated stimuli (Yakovlev and Faden, 2001; Knoblach et al., 2002). Although several caspases seem to be involved in post-traumatic apoptotic cell death in the CNS (Knoblach et al, 2002), caspase-3 appears to be the major effector of apoptotic neuronal death (Yakovlev and Faden, 2001). Having demonstrated that caspase-3 activation was significantly decreased in spinal cords of quercetin-treated animals, we feel that this strongly suggests that quercetin significantly reduces the extent of post-traumatic apoptotic cell death after SCI.

Both mechanisms, myeloperoxidase activity in stimulated neutrophils and apoptotic cell death after spinal cord injury, have been observed in material from human patients. Having shown that quercetin, acting neuroprotective in our animal models, interferes with those two pathways, we would assume that quercetin would also be beneficial when administered to human patients in the settings of acute traumatic spinal cord injury and head trauma.

Khaled and colleagues (2003) have demonstrated in an animal model that the state of solubility of quercetin significantly influences both the rate of absorption and the overall amount of absorption after oral administration. Peak values of serum quercetin levels, measured by HPLC, were seen slightly earlier when quercetin was administered in solution

(2 hrs for solution vs. 3 hrs for suspension). Although none of the experimenters has, to our knowledge, studied the pharmacokinetics of quercetin after intraperitoneal administration, we would assume that a similar delay in the availability could be observed for quercetin in suspension with this intraperitoneal administration. Since we have used intraperitoneal administration of quercetin throughout all of our experiments, we might be able to increase availability of quercetin by introducing a solvent that allows us to administer the compound in solution rather than in suspension. On the other hand, considering the not so favorable results from both experimental groups that received an increased amount of quercetin with thrice daily application in the early phase of spinal cord trauma, an increase in the overall available dose might not be desirable. The slow release from a suspension and the lower bioavailability might prove a therapeutic advantage rather than an obstacle. If oral administration of quercetin were considered, it might be worthwhile to explore the usefulness of α G-rutin, a more water-soluble form of the compound (>1 g/ml for α G-rutin vs. 2.4 μ g/ml for quercetin and 51 μ g/ml for rutin). As Shimoi and colleagues have demonstrated, it was absorbed and metabolized more efficiently than either quercetin or rutin, and plasma levels peaked at about 8 hr after ingestion, as seen with quercetin (Shimoi et al., 2003).

10.4 The second hypothesis is proved false

We hypothesized that, while administration of quercetin in the acute phase of CNS injury resulted in neuroprotection and functional recovery, no such benefit would be seen with delayed treatment onset.

Against our expectations, recovery of motor function in initially paraplegic animals was seen even when treatment onset with quercetin was delayed for 2 weeks after injury. Although this was unexpected, reasonable explanations with regard to the acting mechanisms of quercetin can be found. Quercetin has been found to inhibit apoptotic pathways, both in vitro and in our in-vivo model. Apoptotic processes have been observed to continue as late as 3 weeks, possibly even years after spinal cord trauma (Johnson et al., 1995; Shuman et al., 1997). The latter would correspond with the clinical observation that, even years after the primary spinal cord trauma, and preceded by a phase of apparent stability, neurological performance of patients may continue to deteriorate. Syrx formation, or increase of the volume of an existing syrx, has been linked to the observed functional deterioration (Hughes, 1988). Apoptotic morphology has been shown to be closely correlated to DNA fragmentation (Schmied et al., 1993). Since quercetin has been shown to prevent DNA fragmentation (Sestili et al., 1998.), it can be expected that even delayed onset of quercetin administration will still prevent some of the late damage. Another explanation for effectiveness of late-onset treatment with quercetin would include the compound's anti-inflammatory capacity. Chronic inflammatory processes result in changes of extracellular matrix composition, which make the environment conducive to scar formation (Fawcett and Asher, 1999). It has been shown that quercetin modulates the extracellular matrix in a way that results in reduced hypertrophic scar formation (Phan et al., 2003). Lavine and colleagues

demonstrated that after administration of a neutralizing antibody to TNF- α , a potent mediator of astrogliosis and cell death, neurological outcome was improved after ischemia-reperfusion injury in a rat model (Lavine et al. 1998). Quercetin has been shown to inhibit TNF- α production (Mastuda et al., 2002; Kahraman et al, 2003). Another indication that quercetin modulates post-traumatic tissue formation has been shown in a model of chronic inflammation, where administration of the compound significantly reduced the extent of granuloma formation (Pelzer et al., 1998).

Thus, quercetin might modulate the environment at the injury site in a way that ultimately prevents scar formation when administered in the appropriate time frame after CNS trauma.

10.5 Conclusions

The results of our experiments suggest that the action profile of quercetin allows the compound to interfere with pathological mechanisms that otherwise would result in significant functional recovery of traumatized CNS tissue structures. Based on the results of our experiments, the following pathways through which administration of quercetin might contribute to neuroprotection are proposed: 1) Quercetin reduces the level of myeloperoxidase activity in injured spinal cord and brain tissue. Less hypochlorous acid is generated in and released from the neutrophils, which prevents damage to adjacent tissue structures. 2) Since less oxidant species are produced, the intracellular pool of reduced glutathione is better preserved, resulting in preservation of structure and function of regulatory proteins and mitochondria. 3) Quercetin decreases delayed apoptotic cell death, as suggested by prevention of the caspase-3 increase seen in injured animals treated with saline vehicle only. Therefore, both early and late administration of the compound should be beneficial, as long as cell death by apoptosis is a contributing factor to secondary tissue damage. 4) Quercetin reduces lipid membrane peroxidation by chelating ferrous iron, therefore preventing the redox cycling of iron ions, which are involved in lipid peroxidation. Since quercetin can be administered in relatively high doses in both animals and human patients without causing significant adverse effects, but neuroprotective effects have been clearly established in animal models of trauma to brain and spinal cord, we feel sufficiently confident to recommend human clinical trials with the compound in the setting of acute traumatic CNS injury.

10.6 Is quercetin fit for clinical trial?

Given the fact that quercetin is a component of most people's regular diet, it would appear that adverse effects to the compound are limited. Therefore, the more relevant question might be rather whether it is reasonable to expect therapeutic effects that warrant time, effort and financial expenses of large-scale human clinical trials. Analyzing the results so far presented in the framework of this thesis, I would propose the following additional investigations:

The experiments investigating the modification of the sequelae of acute head trauma by quercetin administration should be expanded to include the following:

1. Behavioral studies should be added to prove that, as has been shown with the spinal cord injury model, administration of quercetin results in improved recovery of neurological function.
2. Modulation of iron clearance from the site of injury was one of the prominent pathomechanisms, which were found to be modulated in a neuroprotective manner by administration of quercetin in the spinal cord injury model. Therefore, it would appear advantageous to demonstrate that this protective mechanism is acting also in the setting of acute head trauma.
3. The characterization of quercetin action in both animal models would benefit from further investigation of the compound's interference with delayed cell death. Since attenuation of apoptotic cell death has been suggested by the decreased extent of caspase-3 cleavage observed during the acute phase of spinal cord injury, we might expect to see similarly decreased caspase-3 activity with quercetin administration after acute head trauma. Furthermore, since the process of apoptosis is believed to be

- ongoing weeks and months, even years after injury, caspase-3 expression should be analyzed in samples from animals which received delayed treatment with quercetin. To verify whether quercetin administration indeed attenuates apoptotic cell death, nuclear fragmentation should be analyzed using, for instance, TUNEL stain.
4. Although the work presented in this thesis was concerned with prevention of secondary injury rather than regeneration, it would be interesting to investigate whether administration of quercetin modulates the process of glial scar formation. A future curative approach to spinal cord injury will most probably combine several different therapeutic approaches, including prevention of secondary injury and possibly transplantation of cells which help bridging the physical gap created by the injury, transplantation of cells which support re-myelination of neurons that lost their proper myelin sheath as a consequence of the trauma, and administration of growth factors or other substances that support regeneration.
 5. It would be interesting to investigate whether quercetin is actually taken up into cells, or whether it stays in the extracellular space while unfolding its neuroprotective action. Of high interest would be knowledge about whether break-down of the blood-brain or blood-spinal cord barrier is required for the compound's action.
 6. It would be highly desirable to find a method by which to relate duration of relevant patho-physiological processes in the laboratory rat to the duration of the comparable processes in human patients, in order to define therapeutic window and optimal time of treatment onset before the onset of human clinical trial.

If the above questions could be addressed and the results from those experiments would further support our impression that administration of quercetin acts neuroprotective after acute neurotrauma, translation into clinical trials should be attempted.

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