EFFECTS OF QUERCETIN ON EXERCISE POTENTIAL AND EXERCISE-INDUCED CYTOKINES IN THE HORSE

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ABSTRACT OF THE THESIS

Effects of quercetin on exercise potential and exercise-induced cytokines in the horse

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Inflammation, the common physiological response to a wide variety of

injuries and irritations, has been studied in one form or another for literally thousands

of years. Most non-steroidal anti-inflammatory drugs (NSAIDS) work by inhibiting

the production of prostaglandins (PG) via inactivation or modification of the

cyclooxygenase (COX) enzyme. Unfortunately disruption of the COX pathway can

have unintended consequences, such as an increase in tumor necrosis factor alpha

(TNF-α) levels. *In vitro* studies consistently show reductions in inflammatory

cytokines, including TNF-α and interkeukin-1 (IL-1), in cultures of immune cells

after treatment with quercetin. Studies run *in vivo* have been less decisive. Hence, our

goal in this experiment was to test the hypothesis that quercetin, in sufficient dosage,

will increase athletic performance in the horse by decreasing exercise-induced

inflammation via inhibition of the production of inflammatory cytokines. Six healthy,

unfit Standardbred mares (~500 kg, age 4-5 years) were assigned to one of two

groups in a crossover fashion. The horses were dosed via a nasogastric tube BID (8

am and 8 pm) with 6 g of quercetin in tap water for 3.5 days. Two hours after the

seventh and final dose, a graded exercise test (GXT) was run. During the test the

treadmill remained at a fixed 6% grade. At T = 0 the treadmill was started at a speed

of 4 m/sec. Each following minute the treadmill speed was increased by 1 m/sec, up to a maximum speed of 11 m/sec. Blood and muscle samples were collected to measure markers of inflammation. There was a small but significant increase in run time when the horses were dosed with quercetin, and recovery time was shortened significantly when compared to water-treated trials. There were exercise-induced increases in plasma TNF-α, IL-1, interferon-γ, granzyme-B (GrB), hematocrit, total protein, glucose, and lactate, but only with GrB was there a significant drug effect in the quercetin group. Intramuscular levels of IL-1 and GrB also increased significantly with exercise, but there was no effect from quercetin treatment. This study provides compelling evidence that quercetin could be useful in enhancing exercise performance, although the mechanism for this enhancement is unclear. While this study alone is not sufficient to recommend quercetin for mainstream usage, it adds to a growing body of literature supporting the drug's effectiveness.

ACKNOWLEDGEMENTS

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Introduction

An abundance of evidence exists pointing to a link between exercise, muscle damage, immune response, and inflammation. This link is established by cytokines: cellular proteins involved in mediating immune response and inflammation in response to infection or trauma. Cytokine effects are often both redundant and synergistic, so attempting to pharmacologically alter or inhibit their activity can be difficult and have unintended side effects. Quercetin, a type of plant-derived antioxidant known as a flavonoid, has shown promise both *in vitro* and *in vivo* in treating immune system-induced inflammation and associated increases in cytokine concentration. As strenuous exercise is often limited by soreness and inflammation, the purpose of this study was to test the hypothesis that quercetin can increase athletic performance and lower cytokine markers of inflammation.

Exercise and tissue damage

Exercise-induced muscle soreness is typically described as either acute (during or immediately following exercise), or delayed onset muscle soreness (DOMS), which typically presents itself 12-48 hours after exercise. DOMS in humans may result from connective tissue and muscle microlesions, muscle spasms, acidosis, and/or inflammation (Coudreuse et al., 2004).

An elevated level of lactate in response to exercise has been described in the horse (Snow and Valberg, 1994), and increased lactic acid levels are associated with muscle fatigue, soreness, cramping, oxygen debt, and anaerobic threshold in humans(Brooks, 1988). Levels of the pro-inflammatory, pro-algesic compound prostaglandin E_2 (PGE_2)

increase in response to exercise, an effect that can be negated in humans by the analgesics ibuprofen and acetaminophen (Trappe et al., 1987).

Increases in creatine kinase (CK) concentrations are commonly associated with muscle damage in humans (Bruunsgaard et al., 1997; Ostrowski et al., 1998b, Toft et al., 2002), although this increase may not be linked quantitatively or even qualitatively with the severity of the damage (Evans and Cannon, 1991). In contrast, other human studies have shown significantly higher CK levels following eccentric (lengthening) muscle contractions when compared to concentric (shortening) contractions (Croisier et al., 1996). Ultrastructural muscle damage, which occurs when muscle cells containing contractile proteins suffer microscopic tears, has been shown to follow eccentric exercise (Evans and Cannon, 1991). It should be noted that this is the same study which did not find a link between CK levels and degree of muscle damage.

Inflammatory cytokines: an overview

The term *cytokine* refers to a variety of pleiotropic cellular proteins involved in mediating both pro- and anti-inflammatory effects. While most cytokines function in an autocrine or paracrine manner (Kaplan, 1996), several may also have systemic effects. Cytokines behaving in an endocrine manner, such as interleukin-1ß (IL-1ß), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), can have far-reaching effects, such as inducing generalized septic shock syndrome in species ranging from mice to humans (Beutler and Cerami, 1987; Akira et al, 1993; Dinarello, 1996). IL-1ß and TNF-α can also have biologic activity when expressed on the cell surface in membrane-bound form (Kaplanski et al., 1994; Schmid et al. 1995).

Cytokines often function in a cascade, their presence inducing activation of other cytokines. A well described model involves lipopolysaccharide (LPS)-induced sepsis in animals (Beutler and Cerami, 1987). LPS induces production of TNF-α, which in turn triggers increased levels of IL-1ß, followed by a rise in IL-6 concentration. If TNF-α is dosed directly without introduction of LPS, the cascade continues normally (Beutler and Cerami, 1987).

Regulation of inflammatory cytokines typically involves two processes. Many cytokines, including IL-1ß and TNF-α, are held in a biologically inert form until activated. For instance, the protocytokine precursor to IL-1ß remains in the cytosol, inactive, until it is cleaved by the protease IL-1-converting enzyme (Hazuda et al., 1990; Cerretti et al., 1992; Thornberry et al., 1992). Alternatively, the cytokine's receptor may be used to control activity. There are two subtypes of IL-1 receptor (IL-1R), one that is biologically active (type I) and one that is not (type II). The type II IL-1R binds IL-1 without initiating a response, effectively limiting IL-1 bioactivity (Colotta et al., 1994). In addition an inactive form of IL-1ß, known as the IL-1 receptor antagonist (IL-Ra), can bind to the IL-R, limiting receptor availability for IL-1ß (Dinarello, 1996).

Interleukin-1 (IL-1)

IL-1 is a pleiotropic cytokine that acts on leukocytes, endothelial cells, hepatocytes, myocytes, adipose tissue, and certain neurons (Dinarello, 1996). There are two active forms of IL-1, IL-1α and IL-1ß, and typically their biological activities are indistinguishable. The primary difference is that while IL-1ß typically acts in a paracrine or endocrine fashion, IL-1α is membrane-bound and rarely found in circulation except

after severe disease, when it may have been released from dying cells (Watanabe and Kobayashi, 1994). The third IL-1 isoform, IL-1Ra, has been previously discussed.

When IL-1ß binds to a type I IL-R, several biochemical events are induced (Kuno and Matsushima, 1994; O'Neill, 1995). One of the most notable is the phosphorylation of phospholipase A_2 (PLA_2) activating protein, which leads to a rapid release of arachidonic acid (Gronish et al., 1994). Arachidonic acid is a fatty acid and a key inflammatory intermediate. The enzymes cyclooxygenase (COX) and peroxidase are involved in transforming arachidonic acid into PGE_2.

Tumor necrosis factor-α (TNF-α)

TNF-α is an inflammatory cytokine produced by many cell types within the body, though primarily by macrophages. There is a fair amount of redundancy between the effects of TNF-α and IL-1, including adhesion molecule upregulation and accumulation of leukocytes in sites of inflammation. Like IL-1, TNF-α initiates several inflammatory pathways, including the formation of the inflammatory lipid PGE_2 via the COX enzyme. The rising PGE_2 levels inhibit further TNF-α release in a negative feedback loop. In the case of NSAID antagonism of the COX enzyme, the inhibition of PGE_2 formation precludes the negative feedback loop from occurring. Thus TNF-α levels rise (Endres et al., 1996; Slomiany et al., 1997; Bertrand et al., 1998), and the other inflammatory pathways triggered by the cytokine, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK), continue to function. Ironically some of the human diseases commonly treated by NSAIDS, such as rheumatoid arthritis (RA), are thought to be caused by overproduction of TNF-α (Camussi and Lupia, 1998).

In addition to its pro-inflammatory effects, TNF-α has been linked to insulin resistance in humans (Saghizadeh et al., 1996). This study reported increased TNF-α concentration in muscle tissue of insulin resistant and diabetic patients. The resistance is thought to be due to inhibition of insulin receptor signaling and down-regulation of the GLU4 glucose transporter (Ferrier, 2004).

As with IL-1, there are two subtypes of TNF receptor (TNFR). Type I TNFR is responsible for the majority of TNF-α activity, including cytotoxicity, cytostasis, and activation of neutrophils and endothelial cells. Type II receptors are found almost exclusively on immune cells, and play a role in the proliferation of T and B cells, induction of NF-κB, and cytotoxicity (Hohman et al., 1990a, b). TNFR also play a role in the regulation of TNF-α. Both receptor types can be released from the membrane, and can be found in interstitial space and in circulation. These soluble TNFR function as TNF binding proteins, effectively reducing the bioavailability of TNF-α (Engelmann et al., 1990).

Interleukin-6 (IL-6)

IL-6 is another pleiotropic cytokine, and is induced by infection, trauma, or immunologic challenge in nearly every human tissue and cell type (Van Snick, 1990; Hirano, 1992; Barton, 1997). While closely correlated with levels of IL-1ß and TNF-α, IL-6 itself is not an inflammatory cytokine. Rather it is referred to as an "inflammation responsive" cytokine (Pedersen and Hoffman-Goetz, 2000), as its concentration rises quickly in response to elevated levels of IL-ß, TNF-α, and LPS (Shalaby et al., 1989). Infusion of IL-6 in humans does cause fever, but other effects of inflammatory cytokines, such as shock or capillary leakage, do not occur (Mastorakos et al., 1993). More recent

studies have corroborated the lack of inflammatory effect (Pedersen and Hoffman-Goetz, 2000; Febbraio and Pedersen, 2002; Starkie, 2003).

A primary role of IL-6 seems to be to induce production of a broad range of acute phase proteins (APP) in hepatoma cells and hepatocytes (Kordula et al., 1991; Tilg et al., 1997). APPs include microbe-inhibiting proteins such as C-reactive protein, serum amyloid A, and fibrinogen, to name just a few. Acute inflammation results in changes in the levels of these proteins (Sehgal, 1990). While IL-1ß and TNF-α stimulate a limited APP subset, IL-6 is primarily responsible for the response. In fact IL-6 knock-out mice are unable to mount an APP response to injury or infection (Kopf et al., 1994).

IL-6 also serves to dampen the inflammatory effect of IL-1ß and TNF-α. The presence of IL-6 induces corticotropin (Lenczowski et al., 1997; Raber et al., 1997; Bethin et al., 2000), which increases synthesis of glucocorticoids in the adrenal gland. Glucocorticoids, in turn, suppress the synthesis of IL-1ß and TNF-α. This effect was demonstrated more recently by Starkie et al. (2003), who showed that IL-6 can inhibit endotoxemia-induced increases in plasma TNF-α concentration. Glucocorticoids also suppress further release of IL-6 (Waage et al., 1990; Amano et al., 1993).

Interferon-γ (IFN- γ)

IFN-γ is an inflammatory cytokine produced primarily by two main cell types: T cells and natural killer (NK) cells (Farrar and Schreiber, 1993; Boehm et al., 1997). It is a part of a signal-amplification system involving the cytokines TNF-α and IL-12 (Bancroft et al., 1991; Tripp et al., 1993). These studies demonstrated that when a macrophage is exposed to bacterial products, it will produce low levels of TNF-α and IL-12. These cytokines then induce IFN- γ production from NK cells, which activates more

macrophages in the vicinity of the infection. These newly-activated macrophages will then produce higher levels of TNF-α and IL-12. This positive-feedback loop facilitates early control of infection (Unanue, 1997). The process is halted by the presence of IL-10 (Tripp et al., 1993), which prevents macrophage release of TNF-α and IL-12. This in turn inhibits NK cell activation and IFN- γ production.

The role of IFN- γ in the immune response cannot be understated. In addition to activating macrophages (Nathan et al., 1983; Buchmeier and Schreiber, 1985), IFN- γ reduces their susceptibility to microbial infection and enhances recognition of targets (Belosevic et al., 1988). Mice treated with anti-IFN- γ antibodies (Buchmeier and Schreiber, 1985; Nacy et al., 1985; Suzuki et al., 1988; Bancroft et al., 1991) or with disrupted IFN-γ or IFN- γ-related genes (Dalton et al., 1993; Huang et al., 1993) die when exposed to normally sub-lethal doses of various microbial pathogens.

Granzyme A & granzyme B (GrA and GrB)

Granzymes are actually serine proteases rather than cytokines, but are included here because of their relationship to inflammatory cytokines. They are released from cytotoxic lymphocytes (CL), including cytotoxic CD8 T lymphocytes (CTLs) and NK cells, in adaptive and innate immune responses, respectively (Berke, 1995a). There have been numerous studies indicating that GrA and GrB are important for the initiation of apoptosis in infected cells (Shi et al., 1992; Smyth and Trapani, 1995; Froelich et al., 1998). GrB rapidly induces apoptosis, whereas GrA acts more slowly via a different pathway (Shresta et al., 1999).

Although typically released into the intercellular space between the CL and target cell (Berke, 1995b; Kagi et al., 1996), soluble granzymes have been discovered

systemically (Spaeny-Dekking et al., 1998). GrA and GrB levels were found to be elevated in the plasma and synovial fluid of patients with RA (Tak et a., 1999), and intravenous (IV) injection of LPS in humans induced significant increases in the concentration of both proteases (Lauw et al., 2000).

Lauw et al. (2000) also found that monoclonal antibodies (MAbs) against TNF and IL-12 greatly reduced LPS-induced secretion of GrA and GrB levels, both *in vivo* and *in vitro*. MAbs against IFN-γ did not alter granzyme levels, indicating that the cytokine effect on the release of GrA and GrB is mediated through an IFN-γ-independent pathway. In addition to being activated by cytokines, GrA has been shown to increase release of TNF and IL-1ß in human monocytes and mouse macrophages (Metkar et al., 2008) and IL-6 and IL-8 release from human lung fibroblasts (Sower et al., 1996). The later is consistent with another study, which found increased concentration of GrB and GrB$^+$ lymphocytes in bronchoalveolar lavage (BAL) fluid in allergen-challenged atopic asthma patients (Bratke et al., 2004).

Cytokines and exercise

Myriad studies have linked exercise to a change in cytokine levels. Plasma concentrations of TNF-α, IL-1ß, IL-1Ra, IL-6, IL-10, and IFN-γ have been shown to increase in response to exercise in humans (Bruunsgaard et al., 1997; Ostrowski et al., 1998a; Ostrowski et al., 1998b; Ostrowski et al., 1999; Moldoveanu, 2000; Kimura et al., 2001; Helge, 2003; Hiscock, 2003). Several factors including type, intensity and duration of physical work have been shown to influence this effect (Ostrowski et al., 1998a, Ostrowski et al., 1998b, Ostrowski et al., 1999; Kimura, 2001). As described in the preceding section TNF-α, IL-1ß, and IFN-γ are all known to cause inflammation and an

increase in algesic compounds such as PGE_2. Concentrations of anti-inflammatory and inflammation-sensitive IL-6, IL-1Ra, and IL-10 would then increase in response to the surge of pro-inflammatory cytokines.

Exercise-induced increases in inflammatory cytokines can result in muscle and body soreness that can affect an athlete's ability to perform at peak potential. A reasonable hypothesis, therefore, is that decreasing the levels of these cytokines would increase athletic performance.

The horse as a human analog in exercise trials

There is ample evidence in the literature that horses make adequate surrogates for humans in exercise trials (Hinchcliff and McKeever, 1996; Farris, 1998; Hinchcliff and McKeever, 1998; McKeever et al., 1998; Kearns et al., 2001; Kearns et al., 2002). Firstly, it has been well demonstrated that horses and humans have comparable cardiovascular, thermoregulatory (i.e. sweating), immunological, endocrine, and inflammatory responses to exercise (McKeever, 1993; McKeever and Hinchcliff, 1995; Horohov, 1996; McKeever & Malinowski, 1997; Farris, 1998; Horohov, 1999; Kohn, 1999; McKeever and Malinowski, 1999; Betros, 2002; Malinowski, 2002). Possibly most important to the issue at hand, horses also have similar upregulation of cytokines during and after exercise (Barton et al., 2003; Donovan et al., 2007). Controlling as many aspects of a study as possible is vital to the validity and repeatability of the experiment. Using a high-speed horse treadmill researchers are able to mimic various human exercise paradigms, from a short but intense sprint to a much longer and slower paced endurance run, in a controlled environment (Bayly, 1987; McKeever et al., 1993; McKeever & Hinchcliff, 1995; Farris, 1998; Kearns & McKeever, 2002; Ainsworth, 2003; Malinowski, 2004).

Quercetin

Quercetin is a flavonoid found in a variety of edible plants, including numerous berries, onions, apples, tea leaves, and broccoli. Recent studies have shown that it may have great potential for treatment of inflammatory disorders. The reported effects of quercetin in the literature are multifold.

In vitro studies consistently show reductions in inflammatory and inflammation-responsive cytokines in cultures of immune cells after treatment with quercetin. Decreases in the levels of TNF-α, IL-1, IL-6, and nitric oxide (NO) are regularly reported (Comalada et al., 2005; Comalada et al., 2006; Kempuraj et al., 2006; Kaneko et al., 2008; Okoko and Oruambo, 2009). Increases in the level of the anti-inflammatory cytokine IL-10 have also been shown, but do not follow a dose-response curve (Comalada et al., 2006). These experiments were performed in a variety of mouse and human cells, including bone marrow-derived macrophages, pulmonary epithelial cells, mast cells, and lymphoma-derived monocytes (U937 cell line), indicating that the results should correlate well between cell types.

In vivo studies have been less decisive. Frequently no effect is noted, which could be due to various circumstances. Obviously insufficient exposure to quercetin due to poor absorption and/or insufficient dosage will result in a negative outcome. Another hypothesis deals with the fact that a rat colitis model is commonly used in chronic inflammation studies. As quercetin is absorbed in the small intestine, little if any of the flavonoid would make it to the colon (Comalada et al., 2005). But recent studies have produced more encouraging data. A study using a *Helicobacter pylori*-induced ulcer

model in guinea pigs found that orally-dosed quercetin can inhibit inflammation, and greatly inhibited neutrophil infiltration (González-Segovia et al., 2008). In a murine asthma model, treatment with the flavonoid reduced the presence of neutrophils, eosinophils, lymphocytes, and macrophages in BAL fluid (Park et al., 2009). In fact, total cell numbers were reduced 10-fold in the quercetin group when compared to control.

Much research has been done attempting to determine quercetin's method of action. It seems clear that the pathway involves nuclear factor-κB (NF-κB), a theory which will be explored more deeply in following paragraphs. NF-κB is both activated by and induces transcription of inflammatory cytokines in a positive-feedback loop. NF-κB normally exists in the cytosol as a heterodimer complexed with the inhibitory protein IκB. External stimuli, such as inflammatory cytokines, bacterial or viral antigens, or lipopolysaccharides (LPS) activate receptors on the cell surface. This begins a cascade in which protein kinase C (PKC) ultimately phosphorolates IκB, causing its degradation by proteasomes. The disinhibited NF-κB complex enters the nucleus, where it induces transcription of specific genes with activation sites "keyed" to NF-κB, including TNF-α and IL-1 (Manning and Anderson, 1994; Baldwin, 1996; Baeuerle and Baichwal, 1997).

In vitro studies have repeatedly shown that quercetin inhibits the phosphorylation and degradation of IκB (Comalada et al., 2005; Comalada et al., 2006; Nair et al., 2006; Kaneko et al., 2008; Ying et al., 2009), which could explain the reduction in levels of TNF-α and other inflammatory cytokines triggered by disinhibited NF-κB. Conversely when the cell surface receptor-induced cascade is bypassed and PKC is activated directly, the effect of quercetin is nullified (Kaneko et al., 2008). The same paper hypothesized an explanation for this occurrence: quercetin disrupts the formation of lipid rafts on the cell's

surface. Lipid rafts accumulate in response to stimulation and mediate signal transduction (Simons and Toomre, 2000). They have also been shown to be essential for TNF-α-induced activation of NF-κB (Legler et al., 2003). The method by which quercetin disrupts the formation of these rafts has yet to be described.

Conclusion

There seems little doubt that relationships between exercise, immune function, inflammation, and cytokines exist. As previously described, conventional treatments for pain and inflammation, such as COX antagonists, may have deleterious side effects. There is a paucity of information concerning the use of other anti-inflammatory compounds to offset the negative physiological results of exercise and possibly improve performance. Therefore the end goal of this study will be to test the hypothesis that quercetin, in sufficient dosage, can improve markers of athletic performance and reduce exercise-induced increases of inflammatory and inflammation-sensitive cytokines (TNF-α, IL-1ß, IL-6, IFN-γ, and the protease GrB) in horses.

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Chapter 2:
Effects of quercetin on exercise potential and exercise-induced cytokines in the horse.

Introduction

Inflammation, the common physiological response to a wide variety of injuries and irritations, has been studied in one form or another for literally thousands of years. Hippocrates is credited with writing about using willow leaves to treat labor pains and inflammation of joints in the 4th century B.C.E., and even then it was already common practice. We now know that willow contains the compound salicin, which once purified and bound to an acetyl group, is better known as aspirin.

Since Bayer first marketed aspirin in 1899, scientists have continued to delve deeper into the causes of inflammation. Most non-steroidal anti-inflammatory drugs (NSAIDS), including aspirin, work by inhibiting the production of prostaglandins (PG) via inactivation or modification of the cyclooxygenase (COX) enzyme. Unfortunately the inflammatory cascade is very complex, and disruption of the COX pathway can have unintended consequences.

For example, tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine produced by many cell types within the body, though primarily by macrophages. TNF-α initiates several inflammatory pathways, including the formation of the inflammatory lipid PGE_2 via the COX enzyme. The rising PGE_2 levels inhibit further TNF-α release in a negative feedback loop. In the case of NSAID antagonism of the COX enzyme, the inhibition of PGE_2 formation precludes the negative feedback loop from occurring. Thus TNF-α levels rise (Endres et al., 1996; Slomiany et al., 1997; Bertrand et al., 1998), and the other inflammatory pathways triggered by the cytokine, including nuclear factor

kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein

kinase (MAPK), continue to function. Ironically some of the diseases commonly treated

by NSAIDS, such as rheumatoid arthritis, are thought to be caused by overproduction of

TNF-α (Camussi and Lupia, 1998). Clearly a method for directly inhibiting the action of

TNF-α and other inflammatory cytokines could produce a variety of beneficial effects.

One possible use for such a treatment would be to combat the deleterious

inflammatory effects of strenuous exercise. Myriad studies have linked exercise to a

change in cytokine levels. Plasma concentrations of TNF-α, IL-1ß, IL-1Ra, IL-6, IL-10,

and IFN-γ can increase in response to exercise in humans (Bruunsgaard et al., 1997;

Ostrowski et al., 1998a, b; Ostrowski et al., 1999; Moldoveanu, 2000; Kimura et al.,

2001; Helge, 2003; Hiscock, 2003). Several factors including type, intensity and duration

of physical work have been shown to influence this effect (Ostrowski et al., 1998a, b;

Ostrowski et al., 1999; Kimura, 2001). Exercise-induced increases in inflammatory

cytokines can result in muscle and body soreness that can affect an athlete's ability to

perform at peak potential. A reasonable hypothesis, therefore, is that decreasing the levels

of these cytokines would increase athletic performance.

In vitro studies consistently show reductions in inflammatory cytokines in

cultures of immune cells after treatment with quercetin, a flavonoid found in a variety of

edible plants including numerous berries, onions, apples, tea leaves, and broccoli.

Decreases in the levels of TNF-α, IL-1, IL-6, and nitric oxide (NO) are regularly reported

(Comalada et al., 2005; Comalada et al., 2006; Kempuraj et al., 2006; Kaneko et al.,

2008; Okoko and Oruambo, 2009). Increases in the level of the anti-inflammatory

cytokine IL-10 have also been shown, but do not follow a dose-response curve

(Comalada et al., 2006). These experiments were performed in a variety of mouse and human cells, including bone marrow-derived macrophages, pulmonary epithelial cells, mast cells, and lymphoma-derived monocytes (U937 cell line), indicating that the results should correlate well between cell types.

In vivo studies have been less decisive. Frequently no effect is noted, which could be due to various circumstances. Obviously insufficient exposure to quercetin, due to poor absorption and/or insufficient dosage, will result in a negative outcome. Another hypothesis deals with the fact that a rat colitis model is commonly used in chronic inflammation studies. As quercetin is absorbed in the small intestine, little if any of the flavonoid would make it to the colon (Comalada et al., 2005). But recent studies have produced more encouraging data. A study using a *Helicobacter pylori*-induced ulcer model in guinea pigs found that orally-dosed quercetin can inhibit inflammation, and greatly inhibited neutrophil infiltration (González-Segovia et al., 2008). In a murine asthma model, treatment with the flavonoid reduced the presence of neutrophils, eosinophils, lymphocytes, and macrophages in bronchialalveoler lavage (BAL) fluid (Park et al., 2009). In fact, total cell numbers were reduced 10-fold in the quercetin group when compared to control.

Drawing upon the above conclusions and conjectures, we have formulated a new hypothesis: quercetin, in sufficient dosage, will increase athletic performance in the horse by decreasing exercise-induced inflammation via inhibition of the production of inflammatory cytokines. First, however, we must demonstrate that measurable levels of quercetin can be detected in the blood and muscle following oral administration of the flavonoid.

Materials and Methods

Pharmacokinetics

Sample Collection:

Two healthy, unfit Standardbred mares (~500 kg, age 4-5 years) were dosed via a nasogastric tube BID (8 am and 8 pm) with 6 g of quercetin in tap water for 3.5 days. Blood and muscle samples were collected just before the first dose, four hours after the 8 am dosing on days 1, 2, and 3, and 0.5, 1, 2, 4, 8, 12, and 24 hours after the dose on day 4.

The blood samples were collected in vacuum tubes containing EDTA and centrifuged for 10 min at 10,000 rpm. 1 ml aliquots of plasma were pipetted into cryotubes, and 100 µl of 10% ascorbic acid (Sigma Aldrich) was added to each tube as a preservative (Erlund et al., 2003). The samples were vortexed and immediately frozen at -80° C.

Muscle samples were taken from the gluteal muscle under aseptic conditions. Four local subcutaneous injections of lidocaine were made around the collection site to minimize discomfort to the horse. The skin and fascia were punctured with a #11 scalpel blade to allow access to the muscle underneath. Samples were collected using a Bergstrom biopsy needle (6 mm, KRUUSE Worldwide Veterinary Supplier, Denmark) at a depth of ~5 cm. 50 – 200 mg of muscle were collected with each sampling and were frozen immediately at -80° C. Following extraction of the sample the wound was closed with sterile suture.

Processing of plasma samples:

To each stored sample containing 1 ml plasma and 100 µl ascorbic acid, 110 µl of 0.78M sodium acetate (NaAc) (Sigma Aldrich) buffer (pH 4.8) was added. The NaAc is used to maintain a pH of 4.8 during hydrolysis of quercetin glucuronides and sulfate conjugates to quercetin. To this end, the NaAc is followed by the addition of 40 µl of ß-glucuronidase/sulfatase (ß-glucuronidase activity of not less than 100,000 units/ml and sulfatase activity of not more than 7,500 units/ml) derived from Helix pomatia, HP-2 (Sigma Aldrich). 20 µl of 100 µg/ml fisetin (Biochemica Fluka) in methanol was added as an internal standard (Chen et al., 2005). The mixture was incubated for 17 hours at 37° C.

Following incubation, each sample was diluted with 2 ml of 0.5M phosphoric acid, vortexed, and centrifuged for 10 min at 2,000 rpm. The resulting supernatant was subjected to a preconditioned Oasis HLB cartridge (1 cc / 30 mg packing, Waters Corp). The cartridge was mounted on a vacuum chamber and preconditioned with 1 ml of methanol followed by 1 ml of 0.5M phosphoric acid. The plasma supernatant was run through the cartridge, followed by 1 ml of 0.5M phosphoric acid containing 5% methanol. These waste fluids were discarded and new tubes were placed in the vacuum chamber. 3 ml of methanol were run through the cartridge and collected in a conical glass tube.

This eluate was evaporated to dryness in a nitrogen drying rack maintained at 30° C. The residue obtained was dissolved in an additional 1 ml of methanol and dried again. The residue from the 2nd drying was dissolved in 20 µl of methanol and vortexed. One-hundred µl of mobile phase (80% methanol, 20% water, pH 3.73) was added and the

sample was vortexed again. The resulting solution was transferred to an HPLC vial for analysis.

Blank samples of plasma were spiked with quercetin to obtain ratios of 10, 100, 200, 400, and 600 ng/ml quercetin/plasma. These ratios were used to construct a standard curve.

Processing of muscle samples:

This process is modified from de Boer et al. (2005).

While still frozen, 50 ±1 mg of muscle were collected from each sample. 0.85 ml of 0.5M NaAc (pH 5.0, containing 28 mM ascorbic acid), 12 µl of ß-glucuronidase/sulfatase, and 20 µl of 100 µg/ml fisetin in methanol were added. The mixture was thoroughly homogenized, then incubated for two hours at 37° C. Following incubation, 0.85 ml of 0.5M phosphoric acid was added. The mixture was vortexed, then centrifuged for 10 min at 3750 rpm. 1 ml of the resulting supernatant was run through Oasis HLB cartridges and the eluate processed as with the plasma samples.

Blank samples of lean ground beef, obtained from a grocery store, were spiked with quercetin to obtain ratios of 1, 2, and 3 ng/mg quercetin/muscle. These ratios were used to construct a standard curve.

HPLC analysis:

HPLC analysis was performed using an HP1100 with diode array detector and Agilent Chemstation software at a UV detection wavelength of 370 nm. An injection volume of 20 µl was used for plasma samples, while 50 µl was used to adequately detect the lower quercetin concentrations in the muscle samples. Flow rate was 0.5 ml/min, with a mobile

phase of 80% methanol and 20% water, pH adjusted to 3.73 with acetic acid. The samples were processed through an HISIL PS phalanx C18 column 5M (250 x 4.6) mm with attached guard cartridge (20 x 3.2 mm) (Nest Group, Inc) maintained at 30° C. All reagents used were of HPLC grade.

Pharmacodynamics

Six healthy, unfit Standardbred mares were chosen for this study. Prior to dosing, a baseline blood sample was taken from each. In a manner identical to the PK procedure above, horses were dosed with either 6 g of quercetin or water (n=3 each) for 3.5 days. Two hours after the seventh and final dose, the graded exercise test (GXT) was run as described below. Following a washout and healing period of 3 weeks the test was repeated in a crossover fashion. Horses that had previously been dosed with quercetin received water, and vice versa.

The GXT was designed to allow for measurement of maximal oxygen uptake and other factors indicative of exercise performance. Horses were fasted for ~12 hrs prior to the final dosing. On the morning of the test each horse was weighed and dosed with the appropriate treatment (6 mg quercetin in 2 L water, or the water alone) at T = -2 hr. At approximately T = -15 min a catheter (Angiocath, 14 gauge) was inserted percutaneously into the left jugular vein using sterile techniques and local lidocaine anesthesia. At T = -5 min the horse stood quietly on the treadmill (Sato I, Equine Dynamics, Inc., Lexington, KY) while baseline calorimetry data and blood samples (15 ml) were taken. Calorimetry data were collected by fitting the horse with an indirect open-flow calorimeter apparatus (Oxymax-XL, Columbus, OH), which took recordings continuously at 10 sec intervals.

During the test the treadmill remained at a fixed 6% grade. At $T = 0$ the treadmill was started at a speed of 4 m/sec. A blood sample was collected during $T = 50 - 60$ sec, and at 60 sec the treadmill speed was increased to 6 m/sec (5 m/sec was omitted). Each following minute the treadmill speed was increased by 1 m/sec, up to a maximum speed of 11 m/sec, and a blood sample was taken during the last 10 sec at each speed. The test ended when the horse reached fatigue, defined as not being able to keep up with the treadmill despite humane encouragement. The finishing speed and the time spent running at the finishing speed were recorded.

Blood samples were also collected once the horse reached fatigue, at 2 and 30 min, and at 1, 2, 4, and 24 hrs after the conclusion of the test. All blood samples were placed into tubes containing EDTA (Vacutainer, Becton Dickson, Inc., Franklin Lakes, NJ) and kept on ice until the conclusion of the trial. A portion of each sample was analyzed in duplicate for hematocrit and total plasma protein concentration using the microhematocrit technique and refractometry (McKeever et al., 1988); the remainder of each sample was centrifuged for 10 min at 10,000 rpm. Resulting plasma was transferred to cryostat tubes and frozen at -80°C. This plasma was later tested for glucose, insulin, lactate, and electrolyte concentrations.

Muscle samples were collected as described in the PK procedure above at 0.5, 1, 2, 4, and 24 hrs after the conclusion of the test. Each sample was divided into 3 sections, which were placed in cryostat tubes and immediately frozen at -80°C. Frozen samples of blood and muscle were sent to Dr. David Horohov in the Department of Veterinary

Science at the University of Kentucky for measurement of mRNA expression for IL-1,

IL-6, TNF-α, IFN-γ, and granzyme-B (GrB).

Real Time-PCR (RT-PCR):

Samples were analyzed using real time-PCR for measurement of mRNA

expression of IFN-gamma, IL-1, IL-6, TNF-α, and GrB. Total RNA was isolated from

the PAXgene tubes using the PAXgene RNA Blood Kit and RNA from tissue was

isolated using phenolchloroform extraction (BioPhotometer). The RT reactions resulted

in 80 µl of cDNA, and each reaction consists of 16 µl of AMV reverse transcription

buffer (5x), 16 µl of MgCl2, 4 µl of dNTP, 1 ml RNasin, 1 ml Oligo dT primer, and 0.5

ml of AMV Reverse Transcriptase in addition to the 1 µg of RNA. Cytokine-specific

cDNA was amplified and quantitated by 'real-time' PCR (ABI 7500HT Fast Real-Time

PCR System), using the Taq thermostable DNApolymerase and primers based on the

sequences for equine cytokines and β-glucoronidase. Specific primers and FAM-labeled

probes for IFN-γ, TNF-α, IL-6, IL-1, GrB, and β-glucoronidase provided as kits (ABI,

Assay-by-Design) were added to 10 ml reactions in 384-well plates, which consisted of 5

ml of TaqMan Gene Expression Master Mix (ABI), 0.5 of primer-probe and 4.5 of

cDNA. The following PCR conditions were employed: 95°C for 10 mins followed by 40

cycles of 95°C for 15 s and 60°C for 60 s, as recommended by the manufacturer.

Differences in RNA isolation and cDNA construction between samples were corrected

using β-glucoronidase as an internal control for each sample (Breathnach et al. 2006).

Relative differences in cytokine mRNA expression resulting from exercise were

determined by relative quantification. Relative quantification provides accurate

comparison between the initial levels of target cDNA in a sample without requiring that

the exact copy number be determined (Livak and Schmittgen 2001). The pre-exercise samples were selected as the calibrator and the change in cytokine gene expression post expression relative to the calibrator was then determined for each sample.

Statistical analysis:

Graphed results are expressed as means +/- standard error of the mean (SEM). For comparison by group and time a two-way ANOVA for repeated measures was used with the *a priori* level of statistical significance set at $P<0.10$. For comparison by treatment group alone, such as in the case of the exercise performance endpoints, a paired, 1-tailed Student's t-test was used, with the null hypothesis rejected if $P<0.10$. This p-value was chosen to reduce type-II error. Post hoc differences were determined using the Tukey test, and correlation coefficients were derived using the Pearson product moment (Sigma Stat 2.0; SPSS Inc., Chicago, IL).

Results

Pharmacokinetics:

The standard curves created to quantify quercetin presence in plasma and muscle were very linear: $R^2=0.9979$ for plasma (fig. 1) and 0.9998 for muscle (fig. 2). In these graphs the X-axis represents the amount of quercetin added to the control sample, and the Y-axis represents the ratio of quercetin to fisetin detected by the HPLC. This allowed for accurate calculation of quercetin presence in the test samples (fig. 3 & 4). Plasma quercetin concentration increased after dosing, peaking at $1 - 2$ hours and returning to baseline levels by 4 hours. Muscle quercetin levels also peaked at about 2 hours, but did not return to baseline levels until 12 hour post-dose.

Pharmacodynamics

Performance and respiratory markers:

There was no difference between the quercetin and control groups in readings of maximal oxygen uptake (fig. 5) or respiratory exchange ratio (fig. 6). There was a small (~5%) but significant (P=0.06) increase in run time when the horses were dosed with quercetin (fig. 7). Recovery time is defined as the period following the conclusion of exercise during which the oxygen debt is repaid, and is also known as the alactic or fast phase of recovery. When the horses received quercetin their recovery time shortened significantly (P=0.02) compared to their water-dosed trials (fig. 8).

Blood chemistry:

While hematocrit (fig. 9) and total protein (fig. 10) levels rose significantly in correlation with exercise intensity (P<0.001 for both measurements), there was no difference between treatment groups (P=0.70 and P=0.61, respectively). Plasma lactate levels showed a marked increase in relation to exercise intensity, peaking 2 min after exercise completion (fig. 11). This increase was independent of treatment. Glucose levels increased slightly during exercise, and rose sharply 2 min after exercise completion (fig. 12). Insulin concentration began to rise following completion of exercise, although large error bars precludes statistical significance (fig. 13). Neither glucose nor insulin showed a response to quercetin. Plasma levels of sodium, (fig. 14), chloride (fig. 15), and potassium (fig. 16) all rose during exercise, returning to baseline levels 30 – 60 min later. Plasma calcium levels remained unchanged during the experiment (fig. 17). None of the measured electrolytes were affected by quercetin dosing.

Cytokines:

IL-1 mRNA increased slightly until 1 hour after exercise; it then peaked 2 – 4 hours after test completion (fig. 18). There was no significant effect from quercetin dosing. The TNF-α mRNA level increased immediately following exercise, returning to baseline levels after 1 hour. It then rose again, peaking at 4 hours and remaining elevated though 24 hours (fig. 19). These increases were neither significant nor related to treatment. The concentration of IL-6 mRNA became slightly elevated 1 – 4 hours post-exercise (fig. 20), but like IL-1 there was no significant increase and no effect from the drug. IFN-γ mRNA levels tripled 2 min after the end of exercise, then fell gradually and remained somewhat elevated through 24 hours (fig. 21). Quercetin may have increased the speed of the decline, but not significantly so. Lastly, GrB levels increased significantly immediately following exercise, and fell gradually over the next two hours (fig. 22). Quercetin had a small but significant effect, lowering GrB levels at all time points.

Muscle cytokines:

Muscle IL-1 mRNA content (fig. 23) increased significantly 1 hour after cessation of exercise in water treated horses. When the horses were treated with quercetin, the increase was delayed until 2 hours post-exercise and rose only half as much as in the water cohort, but this was not statistically significant. Muscle GrB levels (fig. 24) rose significantly 1 hour after exercise, but there was no effect of quercetin. Levels of TNF-α, IL-6, and IFN-γ mRNA were below the threshold of detection for the test.

Discussion

Pharmacokinetics

This study demonstrated that ~12 mpk of quercetin BID can result in measurable levels of the compound in both plasma and muscle. There did not, however, appear to be any benefit from multi-day BID dosing. The relatively quick clearance of quercetin from both plasma and muscle indicates that dosing every 12 hours is insufficient to maintain elevated concentrations. The highest plasma quercetin concentration achieved in this experiment was 727 nM, considerably less than the 25 – 100 μM used in most *in vitro* experiments showing an effect of quercetin on cytokine levels (Comalada et al., 2006; Kempuraj et al., 2006; Nair et al., 2006; Kaneko et al., 2008; Okoko and Oruambo, 2009).

Pharmacodynamics

Exercise and aerobic performance:

The fast phase of the VO_2 recovery curve involves repayment of the oxygen debt and typically occurs within the first 2 minutes following exercise (Margaria et al., 1933; Piper and Spiller, 1970). We found that when pre-treated with quercetin as opposed to water, the length of the horses' fast phase recovery was shortened by about 20%. While this may indicate a potential for decreased time between intensive bouts of exercise, the lack of a quercetin effect on plasma lactate levels could counter this.

Horses were also able to run significantly longer when dosed with quercetin. Only one of the six horses failed to increase its run time; the others increased by $6.9 \pm 2.3\%$. A recent study using quercetin dosing in healthy-but-untrained humans also showed a

substantial 13.2% increase in time to exercise fatigue (Davis et al., 2010). As ours was a graded exercise test, the strenuousness of the exercise was increased in a stepwise fashion. This caused a clustering of the run time data at the later, higher speeds and likely reduced the amplitude of the quercetin effect. A similar test wherein horses run until exhaustion at a constant speed may give more precise and representative results.

Cytokines:

Several factors including type, intensity, and duration of physical work have been shown to influence the effect of exercise on cytokine levels in humans (Bruunsgaard et al., 1997; Ostrowski et al., 1998a, b; Ostrowski et al., 1999; Moldoveanu et al., 2000; Kimura et al., 2001). This effect, however, is not straightforward. For instance, while some experimenters found TNF-α to increase following exercise (Espersen et al., 1990; Moldoveanu et al., 2000), others found no such increase (Rivier et al., 1994; Ullum et al., 1994; Ostrowski et al., 1998a, b). The same discrepancy can be found in studies measuring concentrations of IL-1, with some studies showing increases (Cannon et al., 1986; Evans et al., 1986) and others showing no change (Ulum et al., 1994; Ostrowski et al., 1998a, b). In a review paper, Pedersen et al. (1998) postulated that these differences are likely due, at least in part, to intensity, type, and duration of the exercise. In the case of IL-6, Brunnsgaard et al. (1997) demonstrated that eccentric-type exercise was much more likely to cause a detectable IL-6 increase than was concentric exercise, and a recent review (Pedersen and Febbraio, 2008) offered convincing evidence that IL-6 levels do not rise significantly unless aerobic exercise, such as bicycling or running, is performed for at least 30 – 60 minutes. As the horses in our experiment only ran 5 – 7 minutes, this may explain why we did not see a significant rise in blood IL-6 levels.

Another cause for discrepancy is the method used for measuring cytokine levels, typically either polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA). PCR indirectly measures gene expression, and is therefore useful for determining up- or down-regulation of mRNA for specific proteins. ELISA, on the other hand, directly measures the levels of the protein. Several cytokines, including IL-1 and TNF-α, are stored in the cytosol in an inactive form, and are released only when cleaved by a protease (Hazuda et al., 1990; Cerretti et al., 1992; Thornberry et al., 1992). In this case, we would not expect to see up-regulation of IL-1 or TNF-α mRNA until the levels in the cytosol were sufficiently depleted. As we used the PCR method of detection, it is therefore unsurprising that we did not see significant exercise-induced increases in blood IL-1 or TNF-α gene expression until 2 – 4 hours after exercise. In support of this hypothesis, one study found that while 3 h of moderate-intensity exercise elevated blood concentrations of IL-1ß, IL-6, and TNF-α as measured by ELISA, it had no effect on their mRNA levels in peripheral blood mononuclear cells as measured via PCR (Moldoveanu et al., 2000). Since both IFN-γ and GrB proteins are produced rapidly in response to an immune challenge, an increase in their corresponding mRNA concentration immediately following exercise is to be expected.

GrB is a serine protease released from cytotoxic lymphocytes (Berke, 1995a, b), and its primary function is to induce apoptosis in infected target cells (Shi et al., 1992; Smyth and Trapani, 1995; Froelich et al., 1998). While there is a paucity of information in the literature directly linking GrB and exercise, a simple indirect link is easily established. GrB levels increase dramatically *in vivo* in response to a bolus lipopolysaccharide (LPS) injection (Lauw et al., 2000). It has been well documented that

LPS levels rise in response to strenuous exercise in humans (Bosenberg et al., 1988; Brocke-Utne et al., 1988; Camus et al., 1997; Jeukendrup et al., 2000), including following a GXT (Ashton et al., 2003). Our measurement of increased GrB blood levels following exercise is therefore consistent with previous experimentation.

Lauw et al. (2000) also demonstrated that anti-TNF monoclonal antibodies (mAbs) significantly reduced the LPS-induced increase in GrB, suggesting that TNF-α is at least in part responsible for GrB release. Transcription of the TNF-α gene is induced by nuclear factor- κB (NF- κB) (Manning and Anderson, 1994; Baldwin, 1996; Baeuerle and Baichwal, 1997). NF-κB normally exists in the cytosol as a heterodimer complexed with the inhibitory protein IκB. External stimuli, such as inflammatory cytokines, bacterial or viral antigens, and LPS activate receptors on the cell surface. This begins a cascade in which protein kinase C (PKC) ultimately phosphorolates IκB, causing its degradation by proteasomes. *In vitro* studies have shown that in both rodent- and human-derived cells, quercetin inhibits the phosphorylation and degradation of IκB (Comalada et al., 2005; Comalada et al., 2006; Nair et al., 2006; Kaneko et al., 2008; Ying et al., 2009). While in theory this sequence could explain our observation of a significant decrease in blood GrB in quercetin-treated horses, the lack of a quercetin effect on blood TNF-α mRNA levels is in conflict with this assumption, as is an experiment in humans that demonstrated no effect of quercetin on ELISA measurements of muscle NF-κB following exercise (Nieman et al., 2007).

GrB has been shown to be locally elevated in BAL fluid in patients with chronic allergic asthma (Bratke et al., 2004) and hypersensitive pneumonitis (Tremblay et al., 2000). Assuming that quercetin is able to reduce GrB levels, via disruption of the NF-κB

pathway or some other method, it could conceivably decrease apoptosis in lung tissue.

This may be partially responsible for the decrease in recovery time observed in this study.

Chapter 3: Conclusion

Acute exhaustive exercise such as a GXT results in significant increases in mRNA transcription of IL-1, TNF-α, IFN-γ, and GrB in the blood, and of IL-1 and GrB in the muscle. Blood levels of IL-6 mRNA were not affected, and muscle levels of TNF-α and IFN-γ were below the level of detection. Multi-day BID administration of quercetin (6g, ~12 mpk) significantly reduced the level of GrB mRNA in the blood following exercise, but had no effect on the other cytokines. This lack of a quercetin effect is consistent with data from human trials (Nieman et al., 2007). When dosed with quercetin the horses were able to run significantly longer (also consistent with human trials: Davis et al., 2010), and took significantly less time to recover following exercise. Maximal oxygen uptake and respiratory exchange ratio were not affected by dosing.

This study provides compelling evidence that quercetin could be useful in enhancing exercise performance, although the mechanism for this enhancement is unclear. Using a higher dosage of quercetin, such that its plasma concentration is more in line with effective *in vitro* levels, could provide a clearer picture. In addition, using an ELISA test to determine protein levels could give a more accurate representation of circulating cytokine levels. As quercetin's method of action is suspected to involve inhibition of cytokine gene transcription, PCR would still be useful in detecting any down-regulation.

If quercetin is consistently demonstrated to increase performance and decrease soreness it would be a boon to anyone who's work or lifestyle demands strenuous activity, including soldiers and athletes of any species. Assuming that its method of action is to disrupt transcription of inflammation-inducing cytokine genes via the NF-κB

pathway, it would avoid most, if not all, of the negative side effects associated with

NSAID use. While this study alone is not sufficient to recommend quercetin for

mainstream usage, it adds to a growing body of literature supporting the drug's

effectiveness.

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Figures
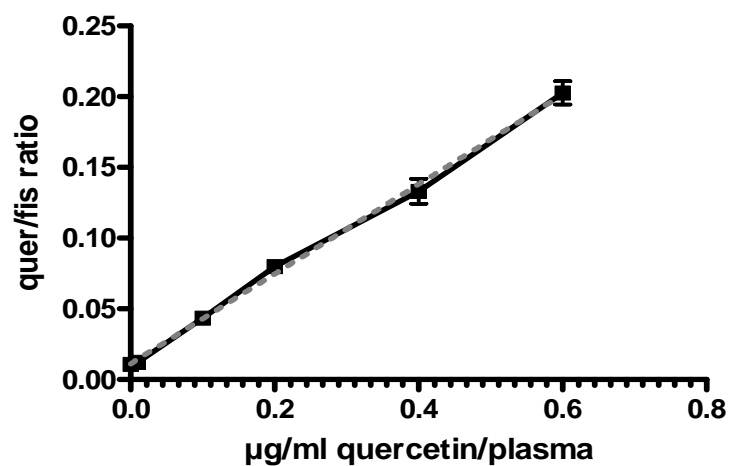
Figure 1: HLPC-measured ratio of quercetin to fisetin vs known concentration of quercetin-spiked plasma samples. R^2=0.9979
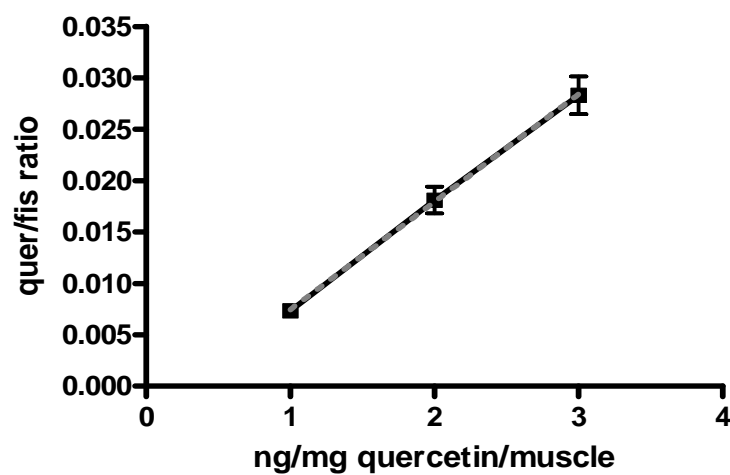
Figure 2: HLPC-measured ratio of quercetin to fisetin vs known concentration of quercetin-spiked muscle samples. R^2=0.9998
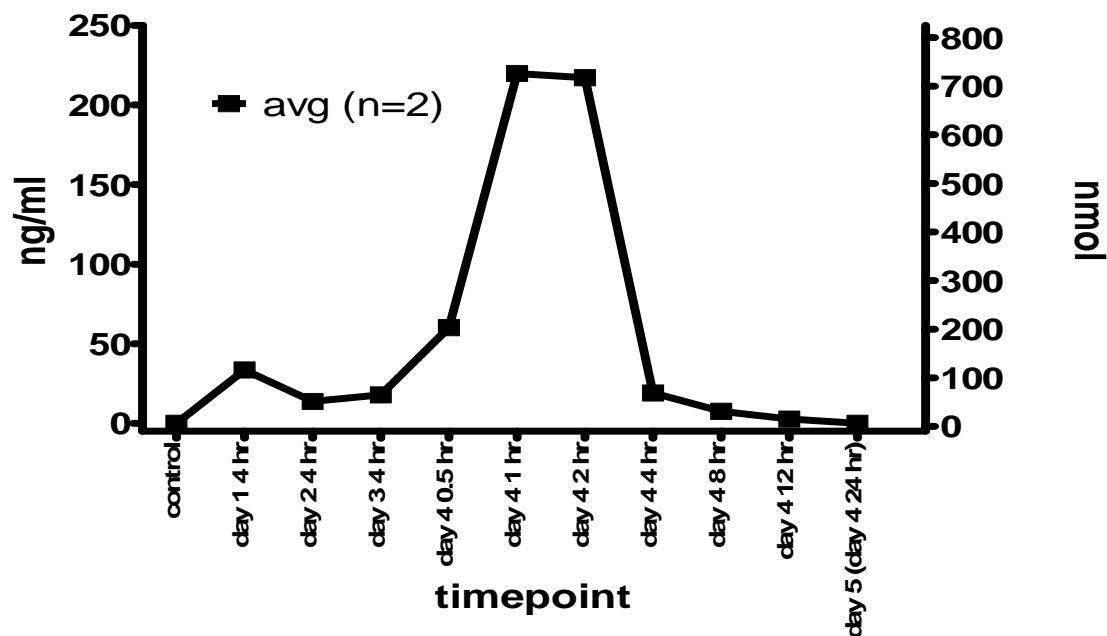
Figure 3: Plasma concentration of quercetin following seven 6g BID oral doses
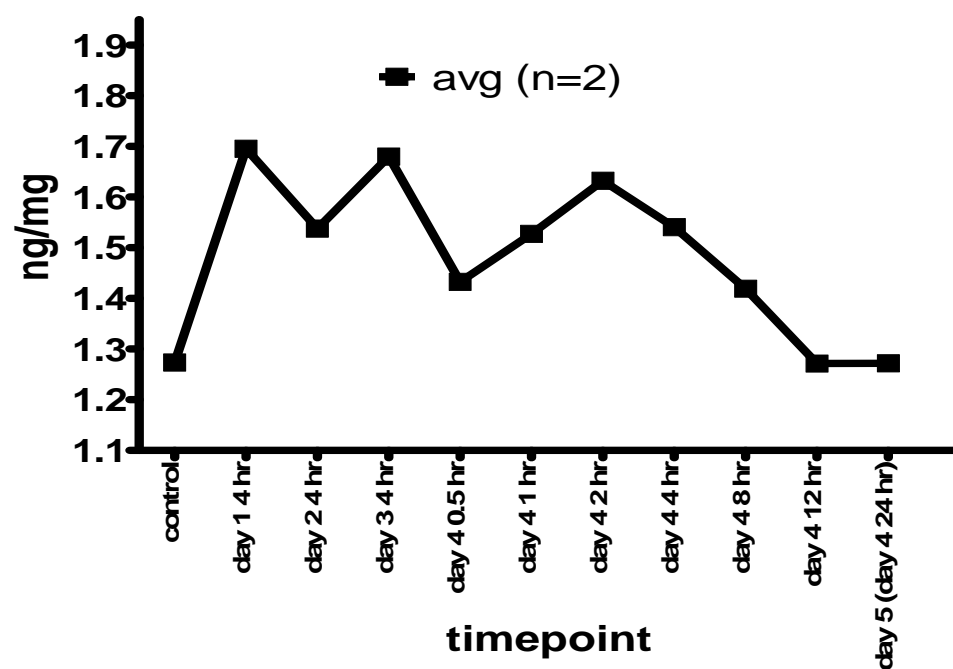
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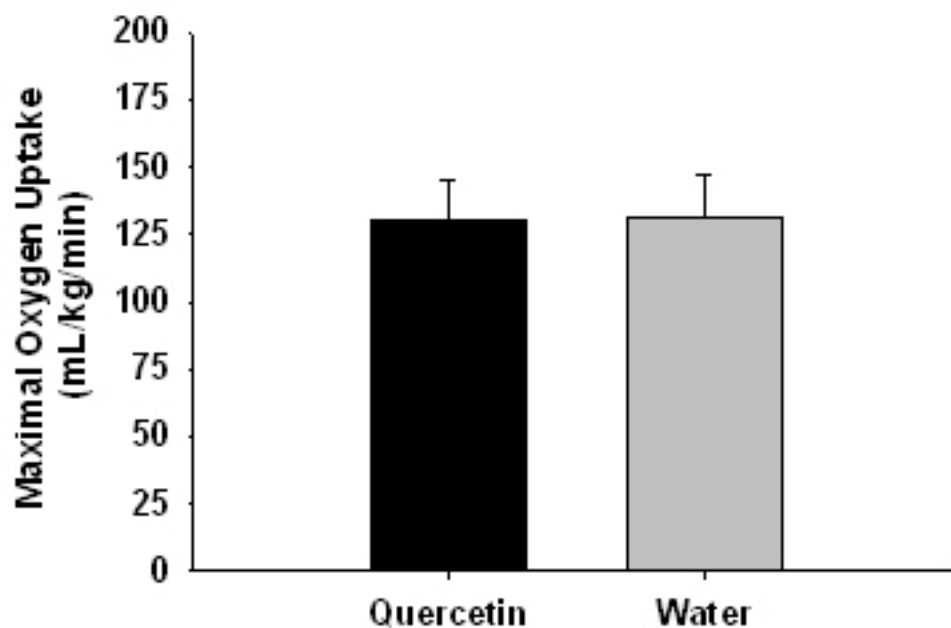
Figure 5: Maximal O^2 uptake during the GXT. There was no significant effect of treatment (P>0.1).
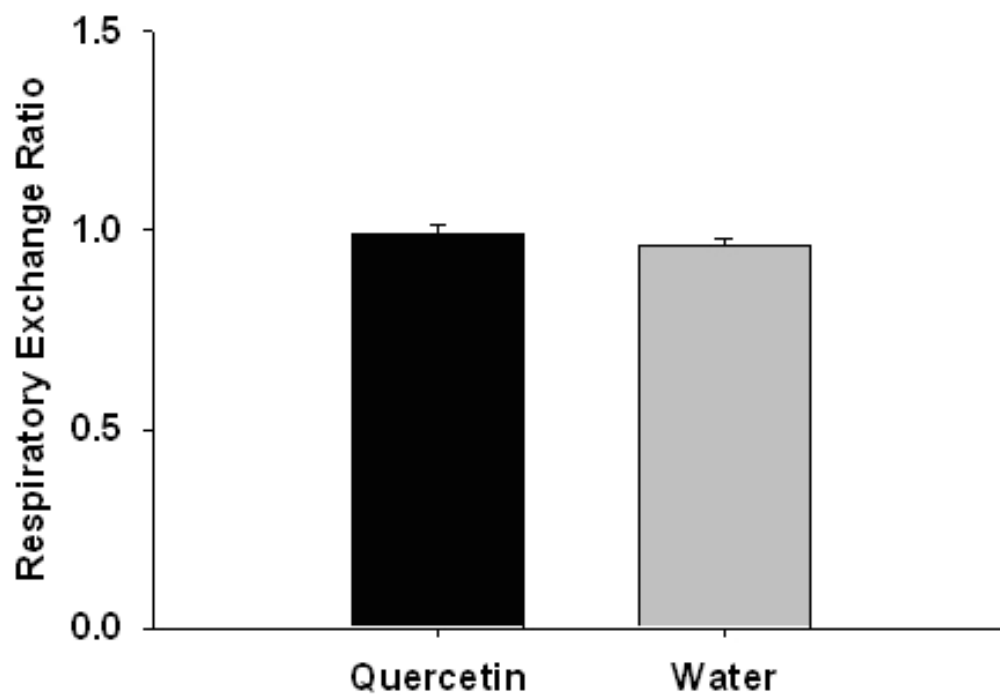
Figure 6: Respiratory exchange ratio during the GXT. There was no significant effect of treatment (P>0.1).
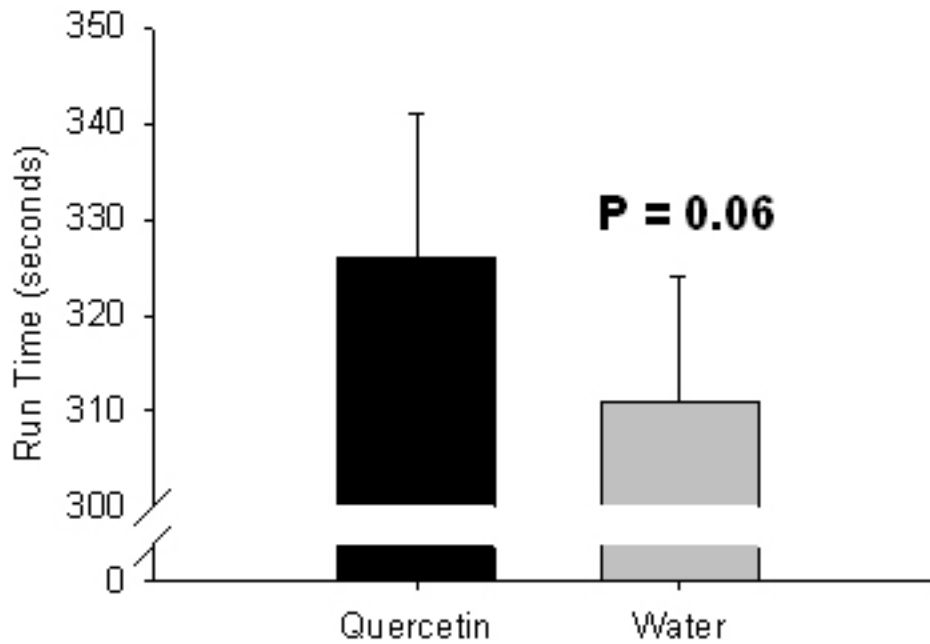
Figure 7: Total run time during the GXT. There a significant (P=0.06) difference between treatments.
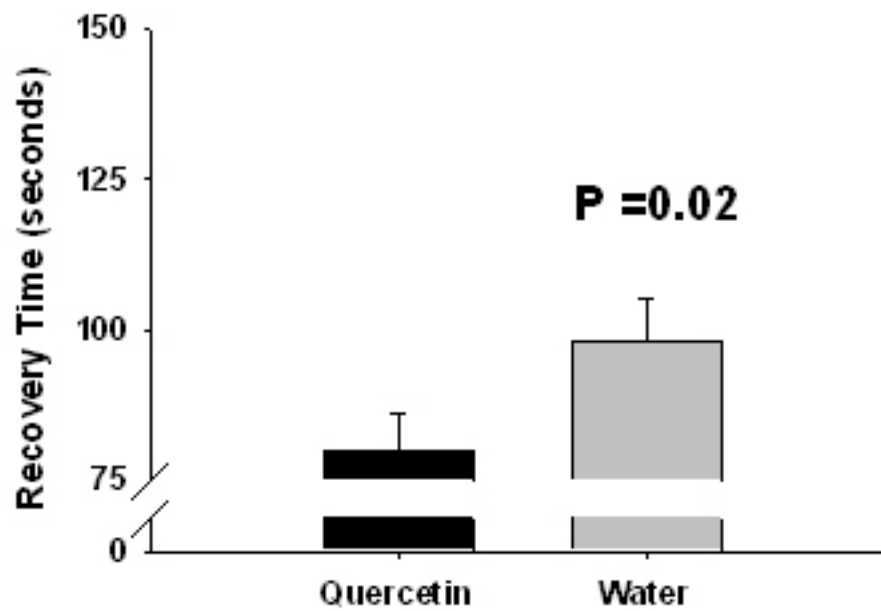
Figure 8: Recovery time (alactic phase) following the GXT. There a significant (P=0.02) difference between treatments.
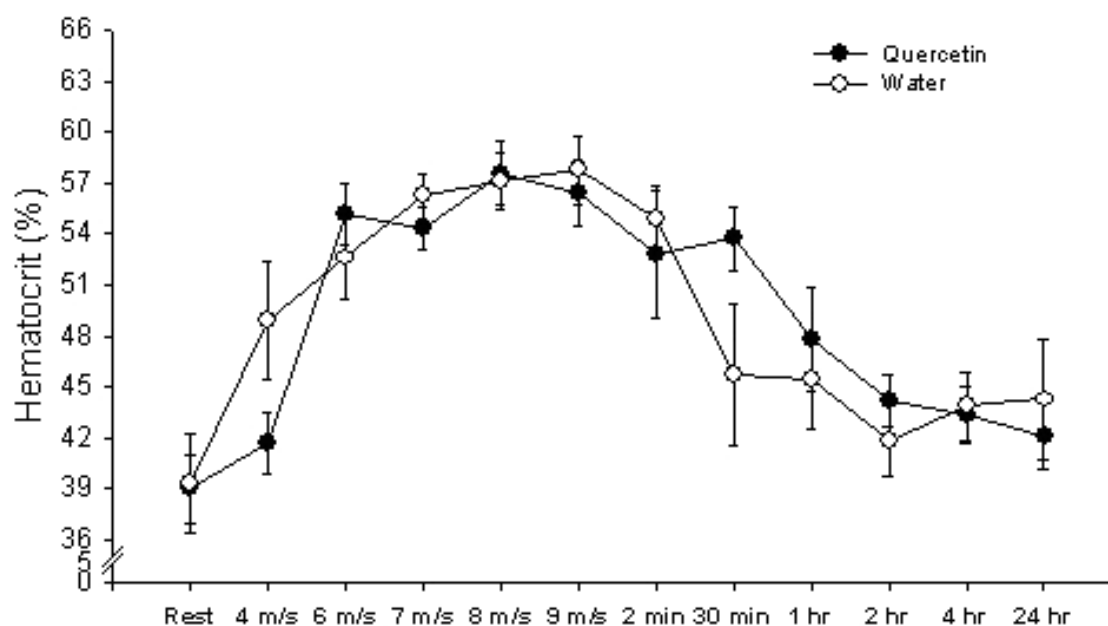
Figure 9: Hematatocrit levels before, during, and following the GXT. There is a significant effect of exercise (P<0.001), but no effect of treatment (P>0.1).
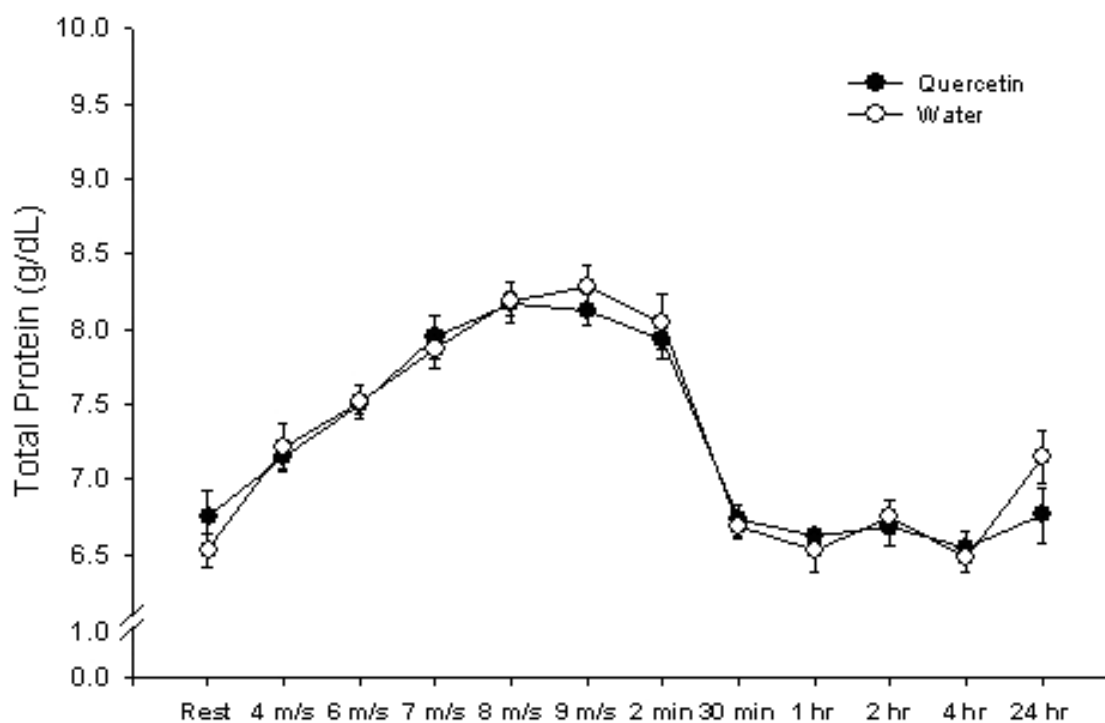
Figure 10: Total protein levels before, during, and following the GXT. There is a significant effect of exercise (P<0.001), but no effect of treatment (P>0.1).
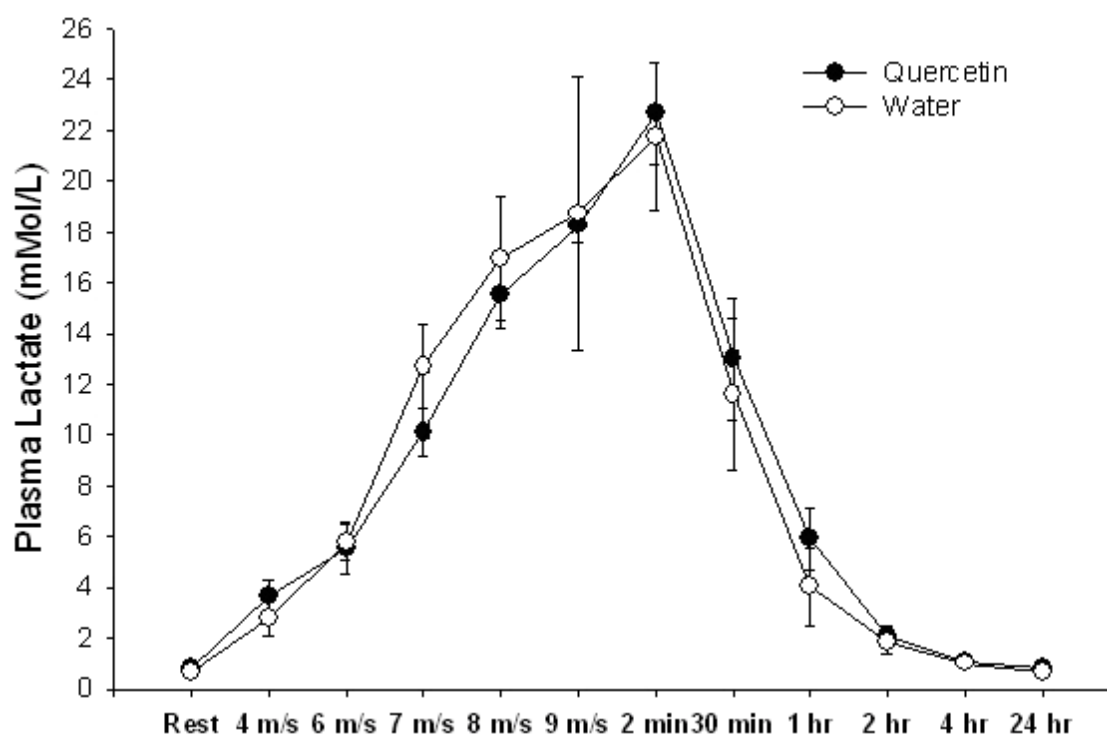
Figure 11: Plasma lactate levels before, during, and following the GXT. There is a significant effect of exercise (P<0.1), but no effect of treatment (P>0.1).
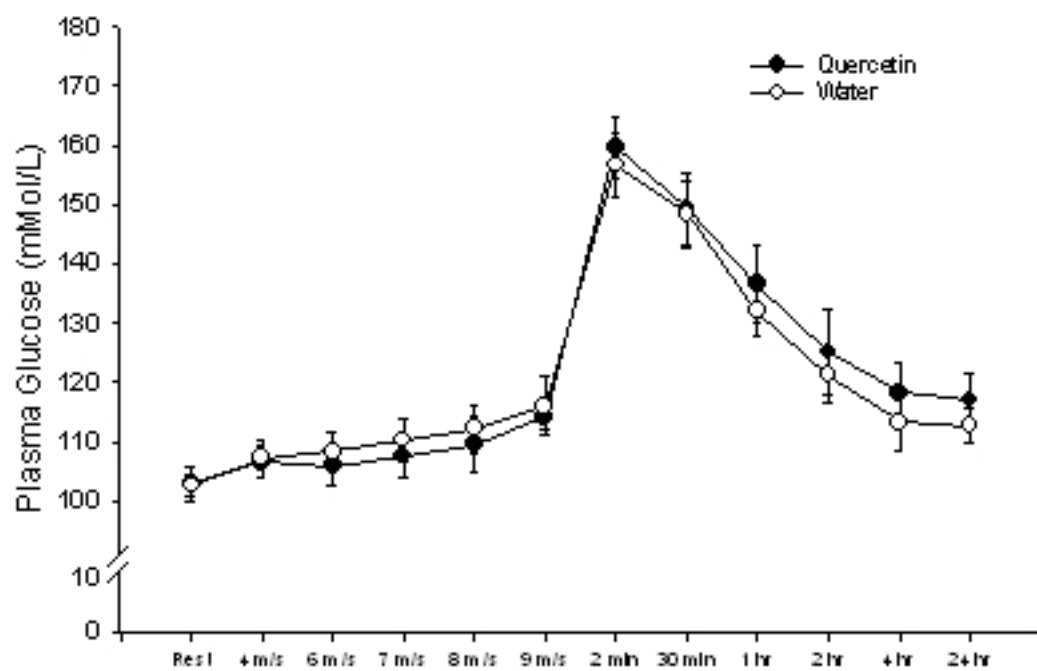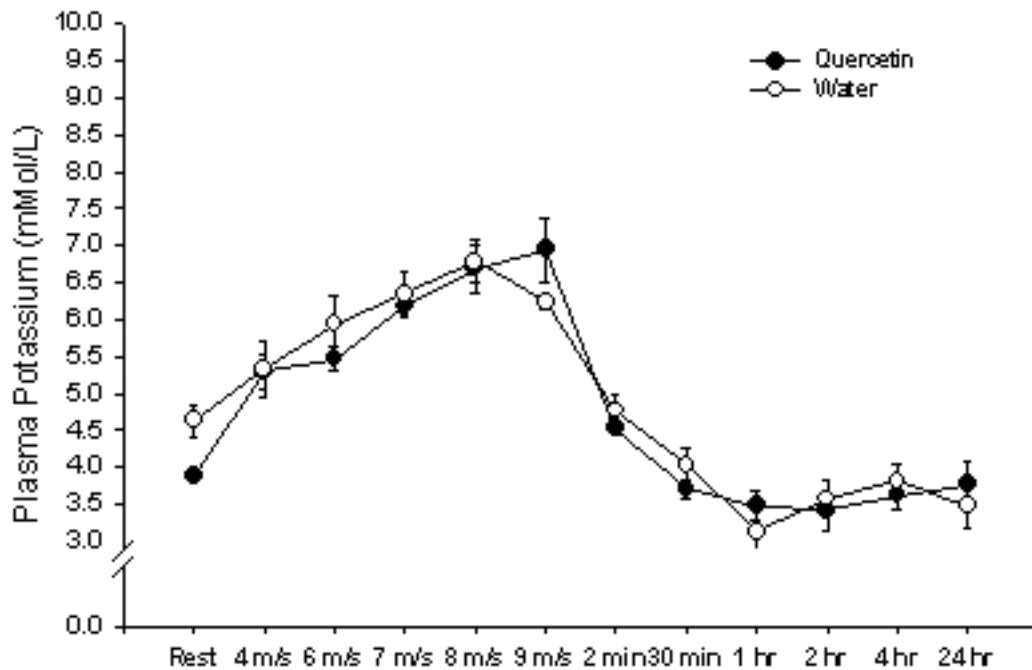

Figure 12: Plasma glucose levels before, during, and following the GXT. There is a significant effect of exercise (P>0.1), but no effect of treatment (P>0.1).
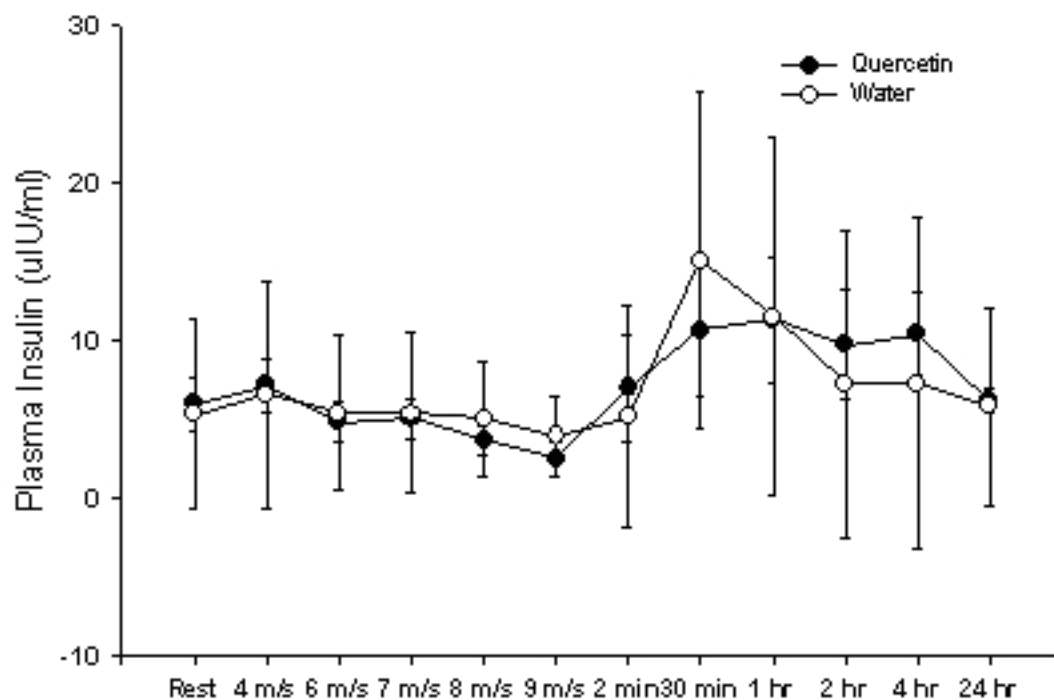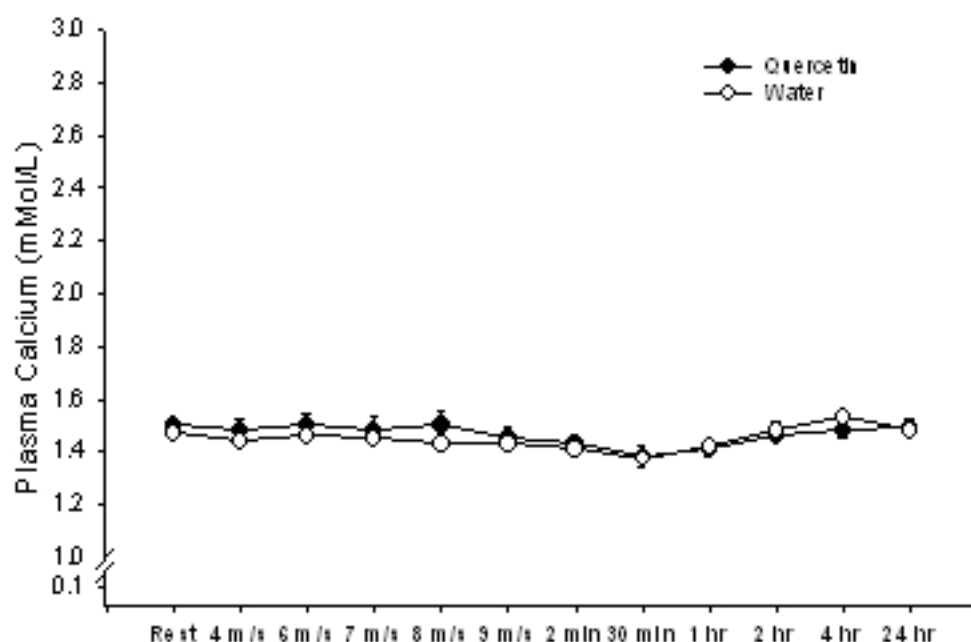
Figure 13: Plasma insulin levels before, during, and following the GXT. There are no significant effects of either exercise or treatment (P>0.1 for both measurements).
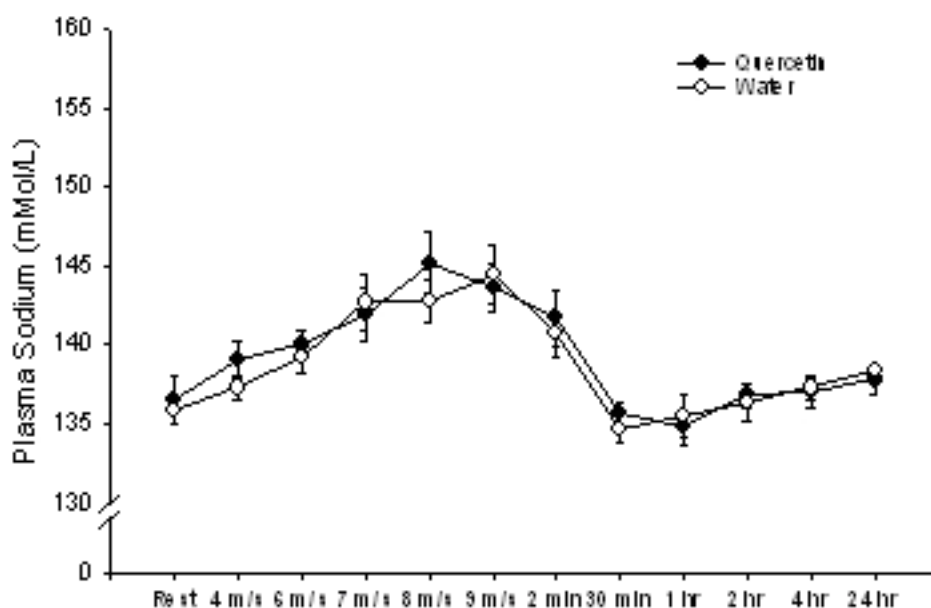
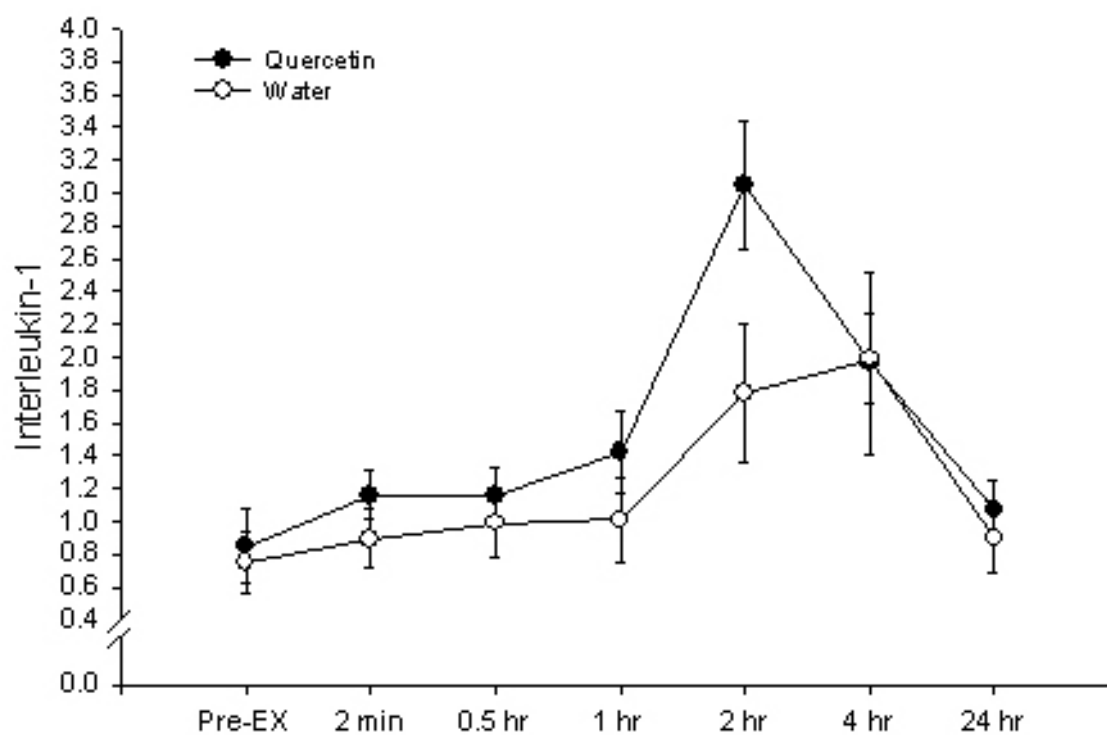Figure 14: Plasma sodium levels before, during, and following the GXT. There is a significant effect of exercise (P<0.1), but no effect of treatment (P>0.1).
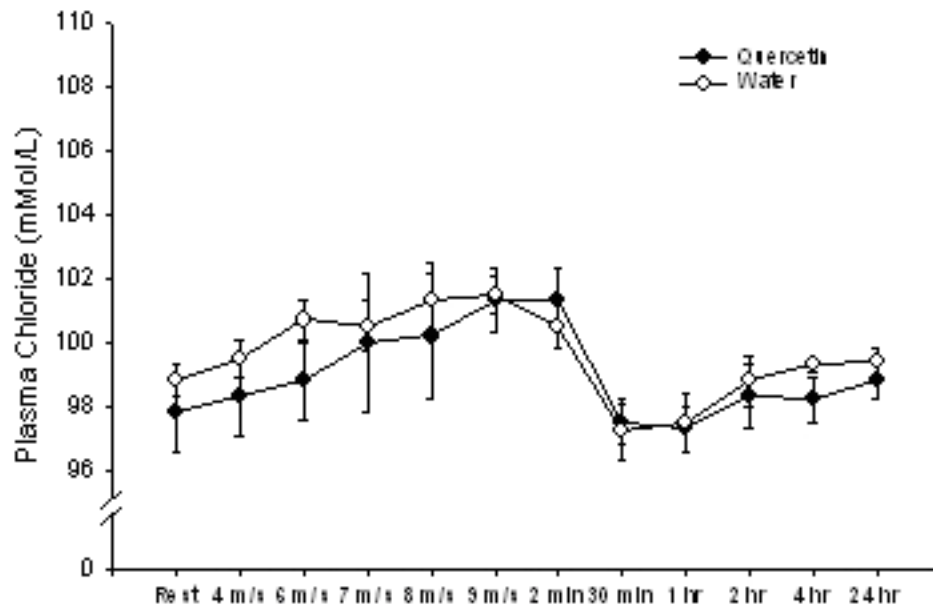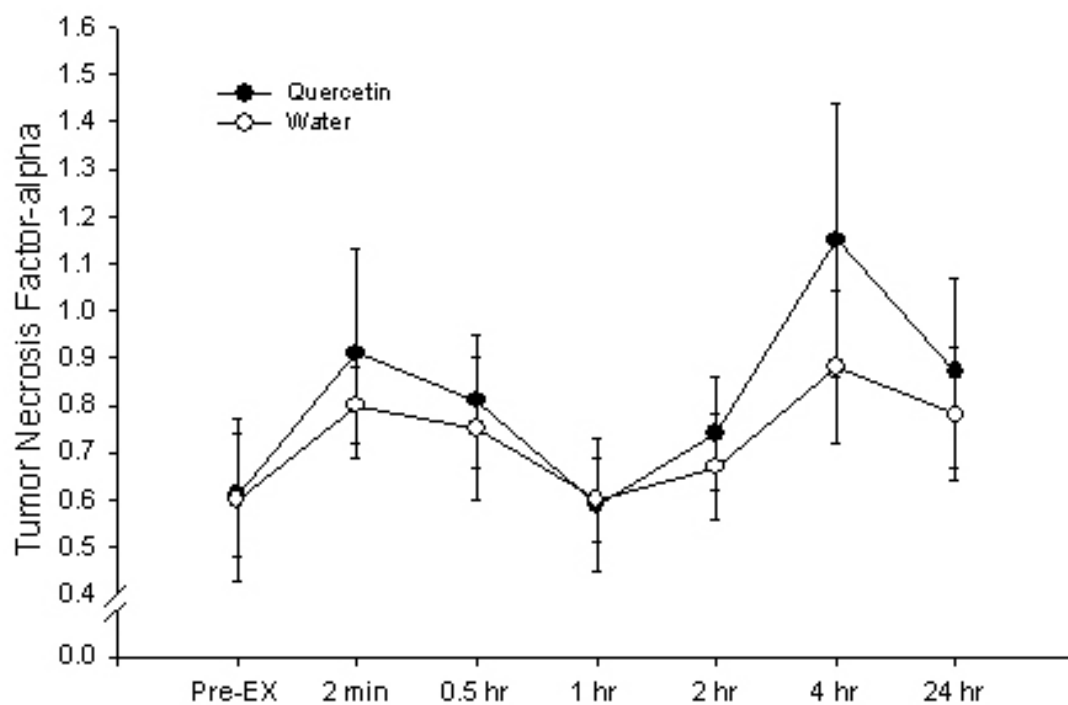
Figure 15: Plasma chloride levels before, during, and following the GXT. There is a significant effect of exercise (P<0.1), but no effect of treatment (P>0.1).

Figure 16: Plasma potassium levels before, during, and following the GXT. There is a significant effect of exercise (P<0.1), but no effect of treatment (P>0.1).
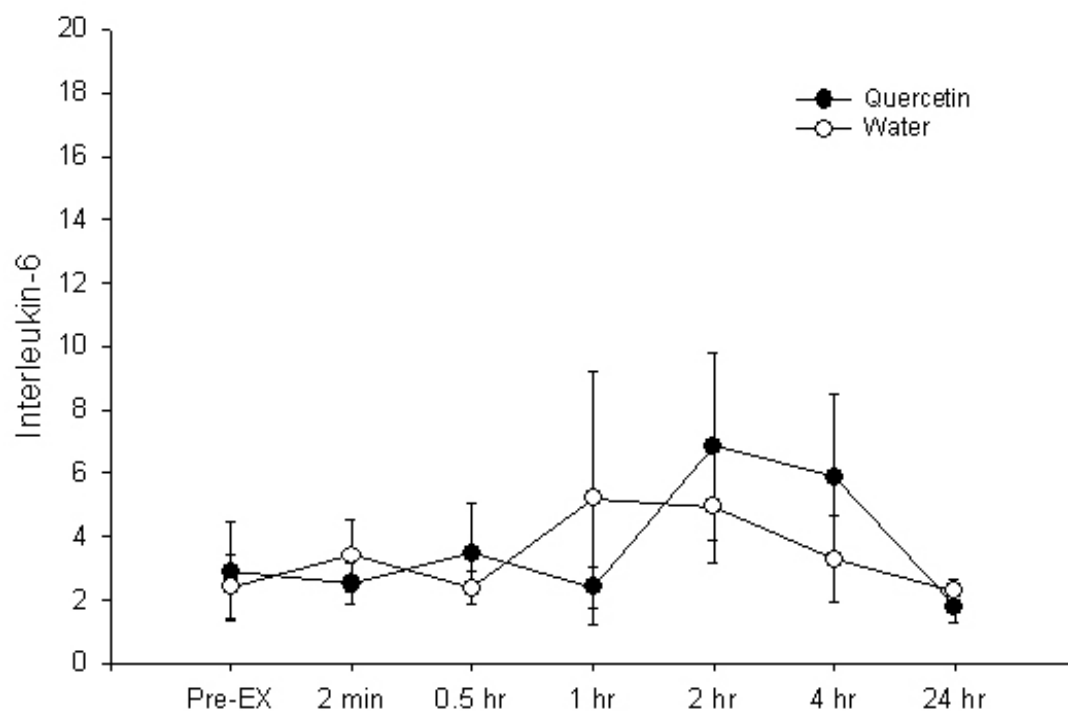
Figure 17: Plasma calcium levels before, during, and following the GXT. There are no significant effects of either exercise or treatment (P>0.1 for both measurements).
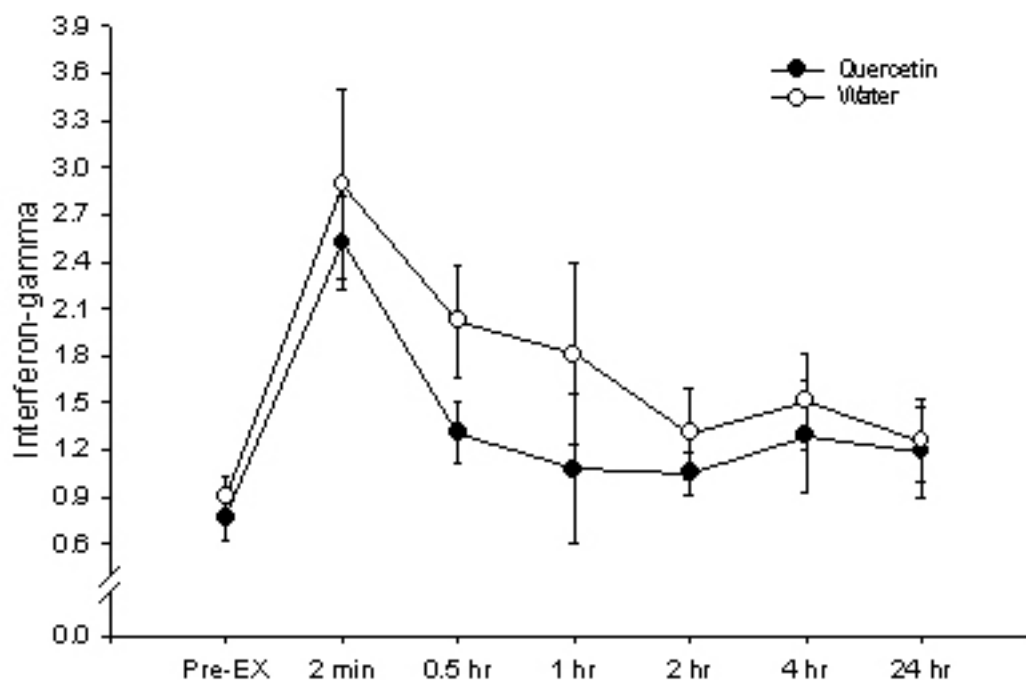

Figure 18: Plasma IL-1 mRNA levels before, during, and following the GXT. There is a significant effect of exercise (P<0.1), but no effect of treatment (P>0.1).

Figure 19: Plasma TNF-α mRNA levels before, during, and following the GXT. There is a significant effect of exercise (P<0.1), but no effect of treatment (P>0.1).

Figure 20: Plasma IL-6 mRNA levels before, during, and following the GXT. There are no significant effects of either exercise or treatment (P>0.1 for both measurements).

Figure 21: Plasma IFN-γ mRNA levels before, during, and following the GXT. There is a significant effect of exercise (P<0.1), but no effect of treatment (P>0.1).
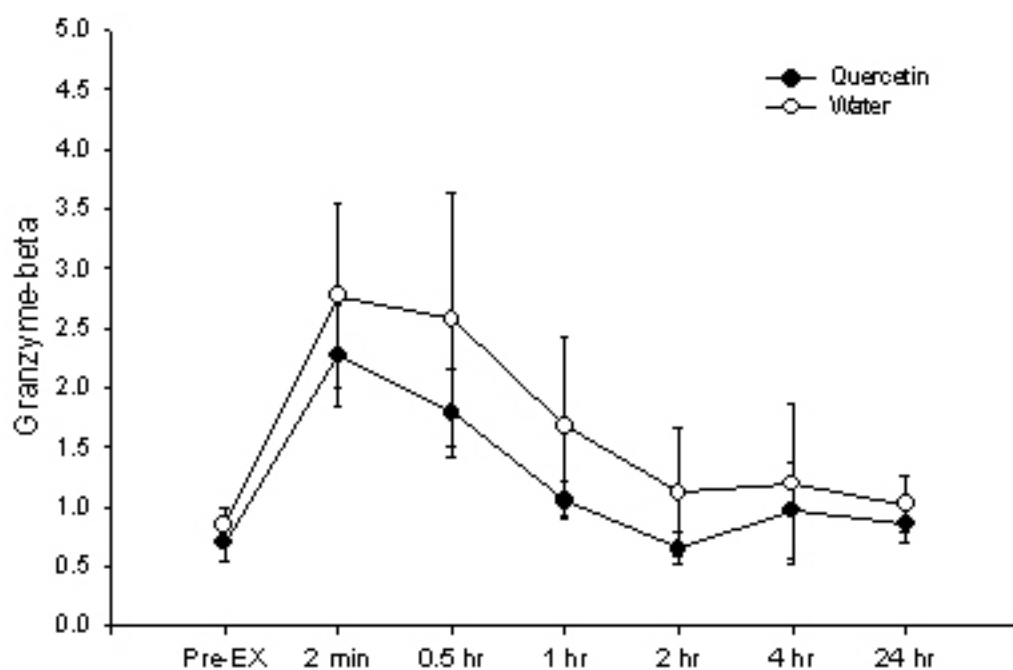

Figure 22: Plasma GrB mRNA levels before, during, and following the GXT. There is a significant effect of exercise (P<0.1), and a significantly lower level in the quercetin group (P<0.1).
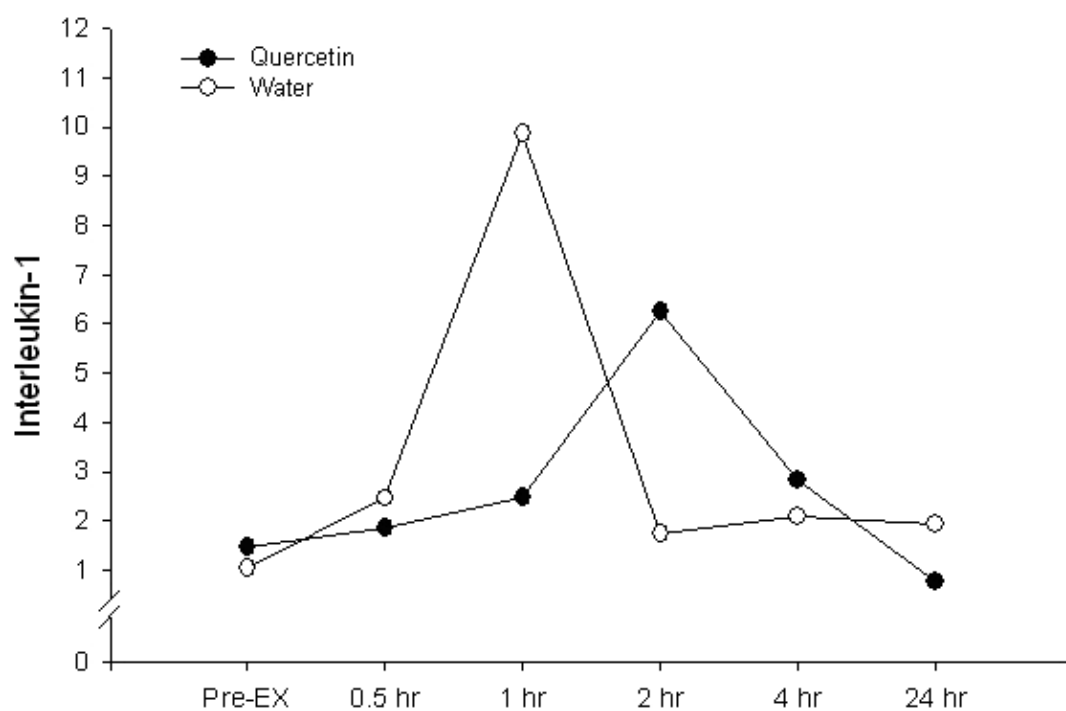
Figure 23: Muscle IL-1 mRNA levels before and after the GXT. There is a significant effect of exercise, ($P<0.1$) but no effect of treatment ($P>0.1$)
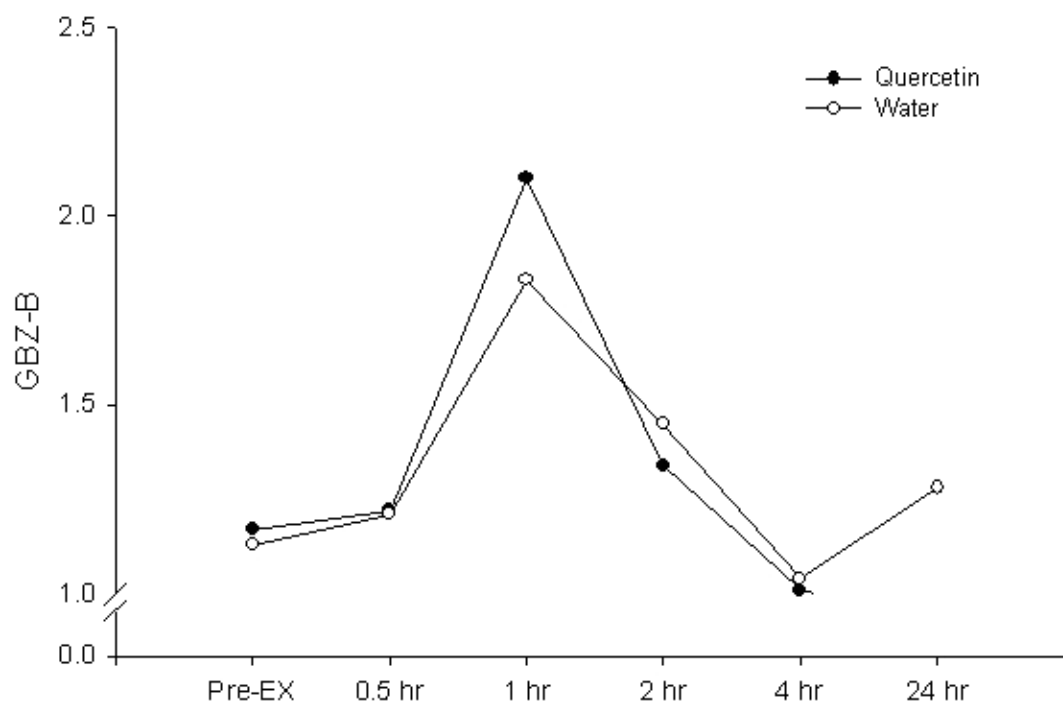

Figure 24: MuscleGrB mRNA levels before and after the GXT. There is a significant effect of exercise, ($P<0.1$) but no effect of treatment ($P>0.1$).