# THE EFFECT OF BODY-WEIGHT SUPPORT TREADMILL TRAINING ON MUSCLE MORPHOLOGY AND GLUCOSE TOLERANCE IN INDIVIDUALS WITH A SPINAL CORD INJURY

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## ABSTRACT

Skeletal muscle is the primary site of glucose disposal in the body, and consequently plays a predominant role in the regulation of blood glucose levels after the ingestion of a meal. Following spinal cord injury (SCI), skeletal muscles below the level of an upper motor neuron lesion undergo marked changes in muscular properties that affect glucose tolerance. Recent studies on individuals with a SCI have reported improved glucose tolerance following periods of electrically-stimulated training. This appears to result from improved muscle morphology and post-exercise insulin sensitivity, and increased GLUT 4 content, enzyme activity, and muscle fiber capillary number. It might be expected that the weight-bearing and greater muscular involvement associated with body-weight support treadmill training (BWSTT) would have an even more profound effect on previously observed responses. The purpose of this study was to investigate whether 6 months of BWSTT improved whole-body glucose tolerance and some of the muscular parameters that are expected to influence glucose metabolism, in a group of 9 individuals with an incomplete SCI. A leg biopsy and a resting, 3 hour oral glucose tolerance test (OGTT) were performed on each subject before and after 6 months of training. Analysis of the muscle biopsy revealed significant increases in post-training muscle fiber size for type I ($P=0.01$) and type IIa ($P=0.05$) fibers in comparison to pre-training values. A significant increase in type IIa fiber proportion ($P=0.03$) and a significant decrease in type IIx fiber proportion ($P<0.01$) were found following training. In addition, significant increases in post-training GLUT 4 protein content ($P<0.01$), citrate synthase protein content ($P<0.01$), and citrate synthase activity ($P=0.01$) were found in comparison with pre-training values. No change was found in the number of capillaries per fiber after

training. Plasma insulin area under the time curve (AUC) during the post-training OGTT significantly decreased ($P=0.04$) in the subjects as a result of BWSTT. Despite the large decrease in plasma insulin concentration, blood glucose AUC also showed a significant decrease ($P<0.01$) during the post-training OGTT, indicating a training–induced increase in insulin sensitivity occurred in the subjects. According to the findings, we can speculate that the increase in insulin sensitivity was a result of the increase in muscle GLUT 4 content in the exercised muscles, possibly in combination with an increase in leg muscle mass, as indicated by an increased muscle fiber cross-sectional area. The results are the first in this field to provide evidence that BWSTT leads to beneficial morphological and metabolic changes in skeletal muscle fibers that improve glucose tolerance.

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## Chapter II

## GLOSSARY OF ACRONYMS

ATPase – *adenosinetriphosphatase*

AUC – *area under the curve*

BMD – *bone mineral density*

BWST – *body-weight support treadmill*

BWSTT – *body-weight support treadmill training*

CS – *citrate synthase*

CV – *cardiovascular*

ES – *electrically-stimulated*

ESLC – *electrically-stimulated leg cycling*

FES – *functional electrical stimulation*

GS – *glycogen synthase*

GT – *glucose tolerance*

GTT – *glucose tolerance test*

HK – *hexokinase*

IGT – *impaired glucose tolerance*

Km – *Michaelis-Menten constant*

MHC – *myosin heavy chain*

MRNA – *messenger RNA*

NIDDM – *non-insulin dependent diabetes mellitus*

OGTT – *oral glucose tolerance test*

SCI – *spinal cord injury*

SDS PAGE – *sodium dodecyl sulfate polyacrylamide gel electrophoresis*

VL – *vastus lateralis*

3-MG – *3-O-methyl-D-glucose*

# 1.0 SPINAL CORD INJURY

Traumatic spinal cord injury (SCI) often results in the partial or complete loss of movement of skeletal muscle below the level of the spinal cord lesion. The majority of spinal cord injuries occur in humans at a relatively young age (101), often leaving the injured individual unable to perform sufficient exercise in order to maintain their muscular and cardiovascular fitness. The low level of fitness, as a result of reduced physical activity, often leads to associated secondary pathologies. For example, numerous morphological, physiological, and biochemical changes occur in skeletal muscle that reduce the rate of glucose utilization in the individual with a SCI. Subsequently, these changes have been related to an increase in the occurrence of non-insulin-dependent diabetes mellitus (NIDDM) in this population (8). Prolonged elevation of blood glucose concentrations, as in poorly controlled diabetes, can result in neuropathy, renal failure, peripheral and cardiac vascular disease, and premature blindness (39;89). Hence, interventions to prevent the injury-associated pathologies are highly relevant.

## 1.1 MUSCLE CHANGES ASSOCIATED WITH SCI

Based on the most common fiber types, human skeletal muscle fibers have been divided into slow (type I) and fast (type II) fibers. In human muscles, type II fibers can be further divided into type IIa and type IIx fibers by histochemical myosin adenosinetriphosphatase (ATPase) staining, immunohistochemistry, or SDS PAGE

analysis of their myosin heavy chain (MHC) composition. The MHC molecule is a motor protein associated with muscle fiber contraction. MHC isoforms interact with actin and influence the fatigability and maximum shortening velocity of contraction, and are responsible for the differences in myosin ATPase activity between the different histochemically defined fiber types (12;13). It is now well established, on the basis of electrophoretic and immunohistochemical studies, that within a given single human fiber there may exist more than one MHC isoform (85). Single muscle fibers composed of multiple MHCs are referred to as "hybrid" fibers. Muscle denervation results in the appearance of hybrid muscle fibers in the months following SCI (99). It is believed that hybrid fibers represent a state of transition in a muscle under new functional demands (5). However, hybrid fibers have also been found under steady-state conditions, perhaps suggesting that the coexistence of different MHC isoforms in single fibers is required to meet the specific functional demands placed on the muscle (76).

The vastus lateralis (VL) muscle, a portion of the quadriceps femoris muscle group, has been the muscle of choice to study because of its mixed fiber type composition, trainability, and accessibility. According to Staron et al. (97), who summarized over 10 years of histochemical and MHC data on biopsies from 150 able-bodied individuals, the VL muscle of both men and women contains approximately 41±12% type I fibers, 31±9.6% type IIa fibers, and 20±11% type IIx fibers, with the remainder being hybrids of these three fiber types. All three major fiber types (I, IIa, and IIx) were shown to be larger in the VL muscle of men. In males type IIa fibers had the largest cross-sectional area (6174 ± 1587 $\mu m^2$), and no significant size differences existed between type I and type IIx fibers. For the women, type I and IIa fibers had similar

cross-sectional areas (4084 ± 895 $\mu m^2$), and both were larger than type IIx fibers. The hierarchy of the percentage of fiber type area from largest to smallest was IIa>I>IIx for the men, and I>IIa>IIx for the women (97).

Simoneau and Bouchard (95) have shown that 15% of total variance in the proportion of type I fibers found within a given muscle sample can be explained by subject sampling and variance in analysis, 45% can be explained by genetic factors and 40% by environmental factors. Therefore, changes in environment including functional demands caused by such factors as innervation and mechanical loading, can alter fiber type composition in skeletal muscle (76;95). Periods of immobilization and denervation due to SCI cause a decrease in skeletal muscle protein synthesis (30) and a reduction in fiber size (15), with a concomitant increase in the content of adipose and connective tissue (70). There appears to be no distinct preferential atrophy of a specific fiber type in paralyzed muscle (67), however, the small percentage of type I fibers in the majority of individuals with a SCI makes it hard to draw definitive conclusions on relative fiber size (70;99;100). A large amount of variation in the structure of muscle after SCI has been reported, both between different studies, and between SCI individuals within a single study (70). Some studies have described odd shaped fibers, with large amounts of adipose and loose connective tissue surrounding them (92). In contrast, other studies have reported muscle from persons with a SCI to appear completely normal (87). Mohr et al. (70) suggest that a relationship may exist between the degree of muscle abnormality and the number of years post injury since they found the most pronounced muscle alteration in subjects with the longest standing injuries (>19 years post-injury). Phenotypically, the long-term effect in individuals with a SCI seems to be a very

pronounced reduction or a complete disappearance of type I fibers and a corresponding increase in the amount of type II fibers. Previous data from individuals with a SCI who were 3 to 20 years post injury, showed that 37±16% of fibers within the VL contained MHCIIx alone, 41±16% of fibers co-expressed MHCIIx and IIa, and less than 1% of the total fibers contained MHCI (2). These results seem to confirm the hypothesis that MHCI is the isoform most dependent on constant and persistent low frequency neural input (2), such as that provided by weight-bearing activity (14).

**Table 1:** *CHANGES IN SKELETAL MUSCLE ASSOCIATED WITH SCI*

| Reference | N | SCI | Time Period | Fiber Type Proportion | | | | Fiber Size ($\mu m^2$) | | | Capillaries | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | I | IIa | IIax | IIx | I | IIa | IIx | 1 | 2 |
| (100) | 6 | Y | A | 40 | 46 | NR | 14 | NR | NR | NR | NR | NR |
| | | | B | 32 | 49 | | 16 | | | | | |
| (15) | 15 | Y | A | 42 | 25 | NR | 33 | 3,104 | 3,872 | 3,269 | NR | 175 |
| | | | B | 44 | 11 | | 45 | 2,272 | 1,723 | 2,207 | | 169 |
| (1) | 9 | Y | A | 1 | 32 | NR | 66 | 3,481 | 3,526 | 4,353 | 1.5 | 404 |

*1= capillaries·fiber$^{-1}$; 2= capillaries·mm$^{-2}$ fiber area; NR = not reported; (100) & (15)- A = 6 wks post injury, B = 24 wks post injury; (1) A = 11 yrs post injury. All biopsies from vastus lateralis.*

### 1.1.1 *TIME FRAME OF FIBER TYPE TRANSFORMATION*

Transformations of the MHC in human muscle appear to be uncoupled from the rapid atrophy that occurs when muscles lose their weight-bearing capacity. Disuse-induced changes in MHC gene expression in humans have been reported to occur relatively slowly in comparison to smaller mammals (76). There is little evidence of transformation from fibers expressing slow MHC to fibers expressing fast MHC during

the first 10 months following a SCI (14). Although the reason for such a slow transition process is unknown, Baldwin et al. (5) point out that it would take several half-lives of a MHCI molecule before the type I protein is markedly depleted in a completely denervated muscle. Considering the MHC molecule has a relatively long half-life (5), a slow transition period from a type I to a type II fiber is not overly surprising. One study examined 2 individuals who had suffered a SCI less than 15 months prior to biopsy of the VL and found no decrease in the proportion of type I fibers (87). The decrease in proportion of histochemically identifiable type I fibers appears to be related to the duration of time following SCI (99) since the individuals who were at least 3 years post injury prior to the biopsy demonstrated a reduced proportion of type I fibers relative to non-injured controls (87). Andersen et al. (2) found that only one subject expressed MHCI in their VL fibers after 12 months of functional electrical stimulation training, and that was the subject with the shortest time since injury (3 years). Muscle specific properties must also be taken into consideration when establishing the time frame for fiber-type transformation in response to SCI (99). Although the MHCI gene will become nearly or completely turned off in response to paralysis in the human VL muscle (99;100), the soleus and tibialis anterior muscles in the same individual will maintain some expression of the gene (99), suggesting that the responsiveness of type I fibers is not only related to the duration of the injury, but also to the muscle origin (4).

## 1.2 REHABILITATION METHODS WITH SCI

Individuals with an incomplete SCI frequently identify independent walking as one of their primary goals following injury (22). Conventional gait retraining requires the patient with a SCI to perform weight bearing, weight shifting and balancing exercises prior to attempts at actual locomotive movement (33). Unfortunately, techniques such as these have proven relatively ineffective at improving reduced glucose tolerance and other medical complications that often arise following a SCI (108), such as reduced bone mineral density (BMD) and cardiovascular (CV) disease. It is estimated that only 24% to 39% of patients with a SCI who undergo conventional therapy are able to walk to some degree by the time their rehabilitation program finishes (6;7). A newer form of rehabilitation involves functional electrical stimulation (FES) training. This method of training is usually performed on a computer-controlled, FES exercise leg ergometer (70). Throughout the training period, the patient with a SCI gradually cycles against higher resistance loads. Although FES training has proven beneficial to participants by improving whole body insulin sensitivity and by increasing the oxidative capacity of muscle, this form of rehabilitation does little to improve walking capability.

Unlike FES and conventional forms of rehabilitation, Body-Weight Support Treadmill Training (BWSTT) is task-specific. Instead of teaching the components of gait as individual skills, which is what occurs during traditional forms of therapy, BWSTT allows the patient with a SCI to improve weight bearing, shifting, and balance in the context of actual locomotive movement. In order for conventional therapy to be successful in improving walking ability, the patient with a SCI is required to support a

given percentage of their body-weight with their legs. Due to the rapid muscular atrophy that occurs soon after the initial trauma, lower limb weaknesses may make weight-bearing extremely difficult, if not impossible. Hence, in BWSTT 40% or more of the patient's weight is supported by a harness during the early stages of rehabilitation (108), and stepping is often accomplished through the passive movement of legs by therapists. After a number of BWSTT sessions, where gradual increases in weight-bearing and balance have occurred, many patients learn to walk unaided on the motor driven treadmill (6;7;107;108). In a study conducted by Wernig et al. (108), two groups of people with chronic SCI, matched for type of injury, time after injury and history of previous rehabilitation were trained either conventionally (n=24) or via BWSTT (n=29) for the same amount of time (mean=3.5 and mean=2.5 months, respectively). Results showed that of 18 subjects confined to a wheelchair, 14 became independent walkers following BWSTT, compared to only 1 following conventional rehabilitation programs. Similar results have been reported elsewhere (27;107), including the maintenance of walking capability 0.5-6 years following the completion of BWSTT (109).

Two recent studies (46;54) reported improved glucose tolerance (GT) in the SCI population following periods of electrically stimulated leg cycling (ESLC). The weight-bearing and greater muscular involvement associated with BWSTT would be expected to have even more profound effects on metabolic factors that influence GT. Therefore in addition to potentially improving the walking ability of individuals with an incomplete SCI, BWSTT would likely provide the individual with a SCI with a number of other health benefits.

Studies exploring changes in CV fitness following aerobic arm cycle exercise in individuals with a SCI have shown little benefit (23). During BWSTT, the person with SCI has not only their legs to move, but their arms are also free to move outside the harness. Due to the partial body-weight support, this form of training also allows individuals to exercise for longer periods than they otherwise would be able to accomplish. For these reasons it seems possible that BWSTT could cause a larger beneficial change in cardiac function than previously reported using other rehabilitation methodologies.

A serious medical complication associated with SCI is the rapid loss of BMD (34). Studies examining the effects of standing (90) and FES (73) have failed to show a significant increase in BMD in individuals with a SCI. Unlike other methods of rehabilitation, BWSTT involves a large weight-bearing component, and therefore is likely to cause a much greater increase in BMD following training. Finally, BWSTT will likely have a pronounced effect on the psychological well-being of individuals previously confined to a wheelchair, simply because it enables them to experience the motion of walking upright; however, this remains to be tested.

**Table 2:** *HYPOTHESIZED CHANGES IN SELECTED INDICES OF HEALTH WITH VARIOUS FORMS OF REHABILITATION IN PERSONS WITH INCOMPLETE SCI*

| *Parameter* | BWSTT | SF | Arm Ergometry | ESLC |
|---|---|---|---|---|
| **Muscle Mass Moved** | Large | Small | Small | Moderate |
| **Glucose Tolerance** | ↑↑ | None | ↑ | ↑ |
| **Bone Mineral Density** | ↑↑ | ↑small | None | ↑small |
| **Cardiac Dimensions** | ↑↑ | ↑small | ↑ | ↑ |
| **Psychological Benefit** | ↑↑ | ↑ | ↑ | ↑ |

*BWSTT, body weight supported treadmill training; SF, standing frame (postural) training; ESLC, electrically stimulated leg cycling; ↑, increases in variable (more arrows indicates greater hypothesized increases)*

## 1.3 THE EFFECT OF EXERCISE ON MUSCLE MORPHOLOGY

In individuals with a SCI, as in able-bodied humans, it is well accepted that increases in muscular activity can result in increases in skeletal muscle oxidative capacity. However, shifts in fiber type, which would involve changing MHC composition, are harder to demonstrate (74). Changes in MHC isoforms with exercise training have been shown to follow a general pattern of sequential transition from fast-to-slow: MHCIIx → MHCIIax → MHCIIa → MHCI (75). To our knowledge, no studies as of this time have examined the effects of BWSTT on muscle morphology. All of our knowledge regarding changes in SCI muscle phenotype following exercise training comes from studies involving electrical stimulation. ESLC for 1 year in 5 subjects with SCI has been shown to induce a switch in VL muscle fibers expressing both MHCIIx and MHCIIa fibers, to a muscle completely dominated by fibers expressing only MHCIIa (2). The final step in the fast-to-slow fiber transition, namely the switching of MHCIIa to

MHCI, has not been demonstrated in the VL muscle of individuals with a SCI following exercise training. It appears that increasing the amount of MHCI in SCI muscle is quite difficult. The lack of evidence showing change in % area of MHCI in SCI muscle following training has led some researchers to believe that fibers containing MHCII are more adaptable to exercise stimuli than MHCI. In a study by Andersen and colleagues (2), complete transition to MHCIIa (91% of total fibers) at some point during the fiber-type transition period is not surprising when one considers that 78% of the muscle fibers in the subjects with a SCI were classified as being either hybrid MHCIIax type, or the pure MHCIIx type prior to training. According to the pattern of sequential transition that the muscle MHC isoform tends to follow with exercise training, one would expect a large majority of the total fibers in the Andersen et al. (2) study to express MHCIIa before a significant increase in fiber composition of MHCI would occur, if this were to occur at all. According to this theory, 1 year of stimulation at 60 Hz, 3 times per week, as in the Andersen et al. (2) study, would not likely be long enough to induce the transition of the highly fatigable MHCIIx fibers across the entire MHC spectrum to the highly fatigue resistant MHCI fibers. A dose-response relationship to electrical stimulation has yet to be tested in patients with a SCI, although the one study to show an increase in type I fibers gave patients with a SCI electrical stimulation for up to 8 hours a day, every day for 6 months (67). Unfortunately a valid comparison on the duration of applied electrical stimulation cannot be made with this study because Martin et al. (67) chose to examine the tibialis anterior, a muscle composed of predominantly type I fibers in able-bodied subjects, instead of the commonly used VL muscle.

A number of studies have shown increases in whole muscle cross-sectional area following different forms of electrically stimulated training in individuals with a SCI (56;96). Changes in whole muscle area via computer tomography (CT) scans are not always concordant with changes in muscle fiber mass, but instead may reflect changes in interstitial tissue mass (70). Therefore it is important for researchers to directly assess muscle fiber area. To our knowledge, no studies have examined the effect of BWSTT on muscle fiber area in the SCI population. However, two studies have examined the changes in muscle fiber area using ESLC training in subjects with a SCI (17;70). Chilibeck et al. (17) found a significant 23% increase in muscle fiber area of the VL after 8 weeks of ESLC. Surprisingly, Mohr et al. (70), who utilized a similar training protocol to the Chilibeck et al. (17) study, involving approximately thirty minutes of ESLC each session, did not find a significant increase in VL fiber area. Even more surprising is the fact that the subjects in the Mohr et al. (70) study averaged a work output of $17 \pm 2$ KJs by the end of the study as opposed to the $9 \pm 4$ KJs averaged in the study by Chilibeck et al. (17). The subjects in the Mohr et al. (70) study trained for 10 months longer than the subjects in the Chilibeck et al. (17) study, and although their protocol called for three exercise sessions per week, the subjects only managed to average two exercise sessions throughout the entire year. Two training sessions per week (70) may not have provided a sufficient training stimulus to induce changes in protein turnover leading to hypertrophy.

**TABLE 3:** *CHANGES IN SCI SKELETAL MUSCLE PROPERTIES ASSOCIATED WITH ELECTRICALLY-STIMULATED TRAINING*

| Reference | N | Protocol | Duration | Proportion I | IIa | IIax | IIx | Fiber size ($\mu m^2$) | Thigh size ($cm^2$) | Capillaries 1 | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (17) | 6 | 30 min.<br>3x/wk | Pre<br>8 wks | NR | No Δ | | | 3,428<br>4,206 | NR | 0.75<br>1.04 | 226<br>248 |
| (70) | 10 | 30 min.<br>3x/wk | Pre<br>12months | 4<br>7 | 33<br>61 | NR | 63*<br>32* | 2,330<br>2,759 | 90<br>101 | NR | NR |
| (2) | 5 | 30 min.<br>3x/wk | Pre<br>6 months<br>12months | 1<br>1<br>0 | 21<br>44<br>91 | 41<br>51<br>5 | 37*<br>3*<br>3* | NR | NR | NR | NR |
| (42) | 4 | 2x30min./day<br>90 days | Pre<br>13 wks | No<br>Δ | Sig.<br>↑ | NR | NR | No Δ | NR | NR | NR |
| (67) | 5 | Up to<br>8hr/day | Pre<br>24 wks | 14<br>25 | NR | NR | NR | NR | NR | 1.7<br>2.2 | NR |

*1= capillaries·fiber$^{-1}$; 2= capillaries·mm$^{-2}$ fiber area; * Signifies MHC analysis; NR, Not reported; Muscle- vastus lateralis, except for (67)- tibialis anterior. All subjects were complete SCI.*

## 1.4 GLUCOSE METABOLISM

Blood glucose concentration is maintained within a tight range by opposing hormonal influences. Following a meal, the rise in blood glucose concentration returns to within the normal range of 4-6 mmol through the actions of the pancreatic hormone insulin. Increases in insulin secretion result in reduced glucose output from the liver and enhanced glucose uptake of insulin-sensitive peripheral tissues, most importantly, skeletal muscle (due to its mass). As a result of its predominant role in the disposal of blood glucose following a meal, it is believed that the improvements in insulin action and glucose tolerance following programs of exercise training result from changes in skeletal

muscle properties that affect glucose uptake (17). These changes in skeletal muscle involve the composition of contracting proteins and the content of metabolic enzymes, membrane transport proteins, and muscle capillaries.

### 1.4.1 *ORAL GLUCOSE TOLERANCE TEST*

Resting oral glucose tolerance tests (OGTTs) are often used to provide an index of insulin sensitivity by comparing insulin concentrations and changes in blood glucose concentration following a standard glucose load (24). At rest, a change in plasma insulin concentration is the most potent stimulus for altering glucose uptake by skeletal muscle. After the ingestion of an oral glucose load, 65-90% of total glucose is utilized or stored as glycogen by skeletal muscle, whereas adipose tissue is responsible for the disposal of less than 10% of orally administered glucose (72). Consequently, insulin sensitivity as measured by an oral glucose tolerance test is, to some degree, a reflection of the skeletal muscle/adipose tissue mass ratio (24). Denervation and immobilization of skeletal muscle caused by SCI tends to decrease the skeletal muscle/adipose tissue ratio (15;17), and therefore a large proportion of the SCI population show decreased insulin sensitivity during OGTTs (17). However it is not possible to calculate with complete accuracy, insulin action from standard resting OGTTs, and therefore results from OGTTs must be interpreted with caution (24). Although the OGTT is supposed to reflect whole-body glucose handling by the total musculature, blood is usually taken from a single forearm vein throughout the entire procedure. In addition, many variables are associated with the muscular system that a standard OGTT does not account for. These variables, which

include insulin clearance and distribution volumes for insulin and glucose, will influence both insulin and glucose levels (24).

### 1.4.2 *GLUT 4*

Glucose transport into skeletal muscle occurs by facilitated diffusion down a concentration gradient across the muscle and t-tubular cell membranes. The muscle cell membrane, known as the sarcolemma, is relatively impermeable to glucose (39). Therefore specific transporters are needed for the facilitated movement of glucose from the interstitial space surrounding each muscle cell, to the cell interior. Insulin-stimulated transport is mediated by a membrane spanning protein termed GLUT 4, which is one of a family of glucose transporters found within muscle and adipose tissue (88). GLUT 1 and GLUT 5 isoforms are also found in skeletal muscle, but in much lower abundance (112). The GLUT 1 protein is found within the sarcolemma and facilitates continuous basal glucose uptake, regardless of insulin and contractions. With a very low capacity to transport glucose, the GLUT 5 isoform acts primarily as a carrier protein for fructose (112).

Unlike other glucose transporters found within muscle, GLUT 4 is the only transporter apparently affected by insulin and contractions, the major mediators of glucose transport activity. Insulin stimulation caused a 6.4-fold greater plasma membrane GLUT 4 content compared with that in the basal state, while insulin-stimulated muscles showed a 7.4-fold greater transport activity than basal muscles (91). This suggests that an increase in glucose transport activity in skeletal muscle caused by

insulin-stimulation can be largely accounted for by sarcolemmal GLUT 4. In addition, studies that have used a photolabelling technique suggest that GLUT 4 translocation is likely the only mechanism by which skeletal muscle glucose transport is increased or decreased (21). In normal muscle, GLUT 4 is recycled between the plasma membrane and intracellular storage pools. Unlike other glucose transporters, approximately 90% of GLUT 4 is sequestered intracellularly in the absence of insulin or repetitive muscular contractions (93). There is evidence of two distinct intracellular pools of GLUT 4 in muscle, with at least one of the pools being able to respond to stimuli other than insulin (81). Although it was once hypothesized that the exercise-induced translocation of GLUT 4 to the cell membrane was caused by increased blood flow and therefore greater insulin delivery to the contracting muscle, many studies have shown that contraction can cause GLUT 4 translocation independent of insulin. For example, when hindlimb rat muscles are contracted in the absence of insulin, GLUT 4 protein concentration increases to a similar amount to that which occurs in the presence of insulin (32). In addition, studies have shown the combination of exercise and insulin to have an additive effect on GLUT 4 translocation to the sarcolemma (32;64) and muscle glucose transport (88;106), lending further support to the hypothesis of two distinct insulin and exercise recruitable pools.

For insulin action, the relay of signaling events begins by insulin binding to the outer $\alpha$ subunit of the insulin receptor, causing the autophosphorylation of tyrosine and serine residues of the inner $\beta$ subunit (103). The phosphorylated receptor also has tyrosine kinase activity, which initiates a cascade of events that leads to the activation of insulin receptor substrate-1, insulin receptor substrate-2, and phosphatidylinositol 3-

kinase (PI 3-kinase), leading to GLUT 4 translocation to the sarcolemma (103). Several studies have shown that these early events involved in insulin stimulated GLUT 4 translocation are not components of the signaling mechanism by which contraction stimulates glucose uptake (39). For example, wortmannin, a PI 3-kinase inhibitor, blocks insulin-stimulated glucose uptake, but does not affect contraction-stimulated glucose transport (64). Calcium, nitric oxide, and adenosine are released from skeletal muscle during contraction, and therefore these molecules have been regarded as possible mediators of contraction stimulated GLUT 4 translocation (39).

Goodyear and Kahn (39) suggest that the exercise and insulin-induced recruitment of GLUT 4 to the cell membrane occurs through the regulation of vesicular exocytosis. Upon translocation from the cytoplasm the GLUT 4 containing vesicles fuse with the sarcolemma. Goodyear and Kahn (39) make this assumption based on the fact that several proteins that are involved in regulated endocytosis or exocytosis in other tissues have also been identified as components of GLUT 4–containing vesicles.

#### 1.4.2.1 *FIBER TYPE COMPOSITION AND PROTEIN EXPRESSION OF GLUT 4*

In rodents, it is well established that the GLUT 4 content is greater in more oxidative muscles than in glycolytic muscles (38;44;66). On the basis of this dependency of insulin-stimulated glucose uptake on fiber type in rodent skeletal muscle, a number of studies have considered such a relationship in muscle from humans (3;47;49). One study showed that the insulin-stimulated increase in glucose uptake was positively correlated with the percentage of type I muscle fibers (113), while another study demonstrated a

significant correlation between insulin resistance and the percentage of type IIx muscle fibers (63). Some studies have even found type I muscle fibers to be more insulin sensitive (determined by GLUT 4 translocation) than type II fibers, and type II fibers to be more responsive (determined by GLUT 4 protein content) to contraction-induced glucose uptake than type I fibers (35). However, not all studies have shown similar results regarding the relationship between fiber type distribution and GLUT 4 content in human muscle. Andersen et al. (3) found no correlation between fiber type and GLUT 4 content, while Daugaard et al. (20) observed no difference in the amount of GLUT 4 in fibers from VL expressing either MHCIIa or MHCIIx. Aksnes et al. (1) studied nine quadriplegic patients with complete lesions of their cervical spinal cord. Surprisingly, despite an almost complete lack of type I fibers and an increase in type IIx fibers in the quadriplegic patients, GLUT 4 protein content in the VL muscle remained comparable to healthy control values. Another study demonstrated very minimal differences in GLUT 4 content between muscles with large differences in fiber composition, indicating that the number of GLUT 4 transporters may not be related to muscle fiber composition as previously believed (21;49).

The finding of fiber type-dependent GLUT 4 expression within rodents amid inconclusive evidence from human biopsies is not too surprising. Unlike rodent skeletal muscle, human muscle contains hybrid fibers consisting of a mixture of all three MHC isoforms (75). This makes the process of selectively studying fibers from a single fiber type in human muscle very difficult.

The plasticity demonstrated by contractile proteins as well as oxidative capacity appear to be the characteristics that are most responsible for the performance of different

functional responsibilities by phenotypically similar muscles (41). The activity of both glycolytic and oxidative enzymes in type I fibers from the rabbit tibialis anterior muscle was found to be 2-3 times higher compared with type I fibers from the soleus muscle (21). Therefore differences in GLUT 4 protein expression may also exist between fibers sharing the same phenotype (as determined by MHC content) but located in different muscles, depending on the pattern of use of the individual muscles in question.

Muscle GLUT 4 protein content may correlate more closely with chronic use and potentially the oxidative capacity of a muscle, than to its histochemically determined fiber type composition (21;55). After two weeks of low intensity exercise in able-bodied individuals, Daugaard and Richter (21) found GLUT 4 to be increased only in muscle fibers that expressed MHCI. According to these researchers (21), the type of low intensity exercise given to their subjects has previously been illustrated to recruit almost exclusively type I fibers. Daugaard and Richter (21) therefore suggested that increases in GLUT 4 protein expression occurred only in the fibers actually recruited during exercise. Hence, it makes sense that the greatest increases in GLUT 4 content will occur in human muscles in which all three major fiber types are recruited during exercise training. The selective effect of training on GLUT 4 expression among different fiber types has only been examined in one other study. In contrast to the study by Daugaard et al. (20), Gaster et al. (35) found no difference in the density of GLUT 4 in type I fibers among age-matched control subjects and athletes whose training predominantly results in recruitment of slow-twitch muscle fibers. They did, however, find a 30% increase in GLUT 4 originating from type I fibers within the VL biopsy sample of athletes. It was concluded

that the 30% increase among athletes in GLUT 4 originating from type I fibers within the VL was solely a function of increased type I fiber volume in the muscle (35).

GLUT 4 content in leg muscle does not necessarily correlate with whole-body insulin sensitivity (1;24;71) because adaptations in insulin action are localized to the specific muscles undergoing the contractile activity during training (25). One study compared the insulin-mediated glucose clearance of a trained leg with that of an untrained leg in the same individuals and found no change in glucose clearance in the non-trained leg (25). This adds support to the theory that the cellular process controlling GLUT 4 expression involves the muscle fiber's activity level. Cortez et al. (19) demonstrated that increasing the amount of muscle that was active during exercise further increased insulin-stimulated glucose uptake. This was accomplished by exercising rats at either a low or high intensity according to $VO_{2max}$. Low intensity training resulted only in an increase in glucose uptake in the red gastrocnemius, whereas high intensity training appeared to have utilized additional leg muscles during the exercise, as evidenced by increased CS activity. This resulted in an increase in insulin-stimulated glucose uptake in not only the red gastrocnemius, but also the plantaris and white gastrocnemius muscles. The only muscle to show an increase in CS activity during both exercise-training intensities, which did not demonstrate a proportional increase in insulin-stimulated glucose uptake was the soleus. However the soleus is normally a very oxidative muscle to begin with, and therefore according to Daugaard and Richter's theory (21), this muscle would already contain a large GLUT 4 content and be highly insulin-sensitive, leaving little room for further improvement.

## 1.5 SCI AND GLUCOSE METABOLISM

Poor glucose tolerance and hyperinsulinemia are well documented in quadriplegic patients during an OGTT (28), even in those who have normal fasting plasma glucose concentrations (54;57;59). Often the lack of physical activity in this population leads to increased area under the glucose and insulin time curve (AUC) during a standard 2hr OGTT. Myllynen et al. (72) showed that patients with a SCI had an index of insulin resistance (glucose AUC x insulin AUC) 7 times greater than healthy mobile controls. Not surprisingly, one study found that 51% of subjects with a SCI had NIDDM (29), while another study found that 22% of the individuals with a SCI examined had clinically-defined NIDDM, compared to only 6% of the age-matched able-bodied controls (9). Inactivity has been demonstrated to result in disorders of glucose tolerance; hence, it follows that individuals with the highest and most complete spinal cord lesions will show the highest frequencies of abnormalities in glucose uptake. Bauman et al. (8) performed OGTTs on four different groups of individuals with a SCI, based on the level and completeness of injury. They found that the group with complete tetraplegia (lesion level C8 or above) had the highest frequency of glucose intolerance and was relatively hyperinsulinemic compared with the incomplete tetraplegic group and the two paraplegic group classifications.

The effect that SCI has on muscle GLUT 4 content is illustrated in studies that have used interventions involving a severe reduction in muscle activity or complete denervation. Immobilization for 35 days in a group of untrained individuals caused muscle GLUT 4 protein content to decrease by 50% (10). Additionally, numerous

studies have demonstrated significant drops in GLUT 4 protein expression in skeletal muscle following periods of detraining (50;68;105). Tabata et al. (98) showed that 19 days of bed rest in previously trained subjects decreased muscle GLUT 4 content by 16%. From these results it is clear that muscle activity plays a major role in the expression of GLUT 4 in muscle.

## 1.6 EXERCISE TRAINING AND GLUCOSE METABOLISM

Several studies have demonstrated that endurance trained individuals have a smaller increase in plasma insulin concentrations and a higher rate of insulin-stimulated glucose disposal than do sedentary people in response to an OGTT (24;46;54;111). Furthermore, during hyperglycemic clamps, in which elevated plasma glucose concentrations are kept constant, insulin secretion is lower in trained versus sedentary individuals (24). In spite of this lower insulin response in trained individuals, they consistently show unchanged or improved glucose tolerance (i.e., area under glucose time curve during OGTT) versus sedentary controls (24).

Decreased adiposity and increased lean body mass as a result of exercise will have an indirect, additive effect on insulin action. However, studies that have controlled for these variables still show improvements in insulin sensitivity due to exercise (39). These findings indicate that endurance exercise causes a blunting of pancreatic β-cell secretion, while concurrently causing an increase in insulin action at the muscle (69). Daily plasma norepinephrine concentrations are more than twice as high in athletes compared with sedentary controls (24). This is due to increased sympathetic activity not

only during exercise, but also during recovery. This increased sympathetic nervous activity is hypothesized to be the signal to the insulin producing β-cells that initiates the adaptation to blunt the insulin response to a given glucose load (24).

Many studies have demonstrated an increase in the expression of GLUT 4 protein following exercise training (20;26;48;51;71;77;98). The type of exercise does not seem to matter with regards to increasing GLUT 4 content, as resistance exercise (98), endurance exercise (20;77), and electrical stimulated contraction (16;70;71) have all produced significant changes. Phillips et al. (77) showed that increased training duration at the same workload results in progressive increases in muscle GLUT 4 protein levels in able-bodied subjects. GLUT 4 content increased by 29% after 5 days of cycling, and increased another 36% after 31 days of cycling (77). The effects of long-term training in individuals with a SCI were examined by Mohr et al. (71) who found that 1 year of ESLC increased GLUT 4 content by 105%. Using a more intense training protocol (7 ESLC sessions/wk) on subjects with a SCI, Hjeltnes et al. (46) found even larger increases (378%) in GLUT 4 expression over a much shorter (2 months) time period.

According to Goodyear and Kahn (39), increased maximal insulin-stimulated glucose disposal in trained human muscle is unaffected by 10 days of inactivity, suggesting that the reduction in insulin action following a period of inactivity is the result of a decrease in insulin sensitivity (GLUT 4 translocation) by the muscle and not a decrease in GLUT 4 content. It is also believed that muscle tissue has more insulin receptors than are required to result in maximal insulin stimulation, since indices of total insulin receptor number are no different in trained and untrained muscle (11). Therefore it is unlikely that the training induced adaptation(s) during maximal insulin stimulation

are due to increased insulin receptor number in the trained muscle. Evidence suggests that the increased rates of glucose uptake in trained muscle during pronounced insulin stimulation are due to increases in muscle total GLUT 4 content and presence at the sarcolemma (44;58;71;85).

Using the non-metabolizable glucose analog 3-O-methyl-D-glucose (3-MG), Ivy et al. (52) examined rates of glucose uptake in the hindlimb muscles of trained and sedentary rats. Maximal insulin-stimulated 3-MG uptake was positively related to GLUT 4 concentration; however the magnitude of the increase in 3-MG uptake 24 hours after exercise was greater than could be explained solely by the increase in GLUT 4 protein (52). This indicates that other factors in addition to GLUT 4, contribute to the increased rates of glucose uptake following training in rats. Leg muscle glucose clearance is calculated as the glucose extraction ratio times blood flow (63), and therefore other variables such as glucose supply, the number of capillaries, the diffusion of insulin from the capillaries to the sarcolemma, and the amount and/or activity of oxidative and/or glycolytic enzymes could contribute to rates of glucose uptake.

**TABLE 4:** *CHANGES IN GLUCOSE TOLERANCE ASSOCIATED WITH SCI AND ELECTRICALLY-STIMULATED TRAINING IN INDIVIDUALS WITH A SCI*

| Reference | N | Intervention | Duration | OGTT results | | |
|---|---|---|---|---|---|---|
| | | | | [glucose] | [insulin] | Other |
| (54) | 7 | 30 min.<br>3x/wk | Pre<br>8 wks | 15% ↓ | 36% ↓ | NR |
| (71) | 10 | 30 min.<br>3x/wk | Pre<br>1 yr | No Δ | No Δ | ISGU<br>21% ↑ |
| (46) | 5 | 30 min.<br>7x/wk | Pre<br>8 wks | NR | NR | ISGU<br>33% ↑ |
| (9) | NR | Quad<br>Para<br>Control | NR | NR | NR | 62% IGT<br>50% IGT<br>18% IGT |
| (72) | 18 | 6 wks post<br>Chronic<br>Control | NR | NR | NR | 6 IRI<br>5.5 IRI<br>1.75 IRI |

*ISGU, Insulin-stimulated glucose uptake (mg/min/kg); IGT, Impaired glucose tolerance; IRI, Insulin resistance index (glucose AUC x insulin AUC); NR, Not reported. All subjects were complete SCI.*

### 1.6.1 *CAPILLARIZATION*

Blood flow to skeletal muscle does not appear to be a limiting factor in muscle glucose uptake (53). Therefore in attempting to explain the positive relationship between insulin action and capillary density, studies have explored the diffusion of insulin across the capillary wall (80). Glucose, with an average molecular radius of 0.44nm, is much smaller than the average capillary pore size of 5 to 20 nm, and hence it can exit muscle capillaries by simple diffusion (45). On the other hand, the active transport of insulin across the capillary endothelium appears to be a fairly restricted process that has been suggested to be rate limiting for insulin action in peripheral tissues (63). Exercise studies have found that an increase in the number of capillaries following training facilitates

increased insulin and glucose transport to the muscle by diminishing diffusion distance (43). The increased delivery of insulin to muscle may help improve glucose tolerance in individuals with a SCI who often have a reduced capillary-to-muscle-fiber ratio (1).

Three previous studies (17;67;84) involving ES training have examined capillarization in individuals with a SCI. Of the three, only one study (17) found significant increases in capillary number per fiber with training (1.04±0.2 capillaries·fiber $^{-1}$ post training), and this was still well below that of able-bodied controls (1.4±2.2 capillaries·fiber $^{-1}$) (17). Any benefits from an increase in capillary number in this study (17) were offset by a proportional increase in fiber area within their subjects with a SCI following training. Capillary density did not significantly increase (17), and therefore the relative diffusion distances for glucose and insulin from the capillary to the muscle would have remained the same.

### 1.6.2 *EXERCISE-INDUCED CHANGES IN SKELETAL MUSCLE PROTEINS IN INDIVIDUALS WITH A SCI*

It appears that the molecular mechanism for improved glucose uptake with endurance exercise training is not only related to the increased expression of GLUT 4, but may also be related in part to the increased expression and activity of the proteins hexokinase II (HK II) and glycogen synthase (GS). Hjeltnes et al. (46) showed that ESLC training in individuals with a SCI induced an increase in HKII and GS content in skeletal muscle. Increases in HKII and GS content were thought to be directly coupled to increased skeletal muscle glucose transport, increased glycogen content, and enhanced whole body insulin-mediated glucose uptake in 5 tetraplegic individuals (46).

After transport into the muscle cell, glucose must be phosphorylated to glucose-6-phosphate by HK before being utilized as fuel or becoming stored as glycogen. This step is crucial for efficient glucose uptake because it maintains the inward transmembrane glucose gradient that drives facilitated diffusion of glucose by GLUT 4. Two isozymes of HK are present in skeletal muscle, HK I and HK II. The predominant HK isoform in human skeletal muscle is HK II, accounting for up to 70% of the total HK pool (62). HK II is of primary interest in relation to glucose uptake since insulin (65) and muscle contractions (61), and possibly glucose itself (62), have been shown to regulate HK II expression and activity. ESLC training in individuals with a SCI resulted in a 25%-150% increase in HK II activity (46;60), and a 204±47% increase in HK II protein expression in the VL muscle (46).

Decreased activity and gene expression of HK II in muscle from NIDDM patients (104) implies an important role for insulin in the regulation of HK II function within human muscle. The effects of insulin on increasing glucose uptake in muscle may be partially explained by the observation that the hormone increases the rate of transcription of the gene for HK II, and this results in an increased abundance of HK II mRNA and increased HK II enzyme activity (5). Insulin has been demonstrated to induce a partial binding of HK to mitochondria (83), that results in a decrease in the Km for ATP and increases the Ki for glucose-6-phosphate, reducing the ability of glucose-6-phosphate to inhibit HKII (83). This would allow the effects of insulin to cause a larger increase in glucose uptake by the muscle.

Glycogen synthase (GS) is the rate-limiting enzyme responsible for glycogen synthesis. Hjeltnes et al. (46) found that ESLC led to a dramatic increase in GS protein

expression (526±146%) in the VL of individuals with a SCI, but had no effect on the basal (non-insulin stimulated) GS activity level. According to the authors, the increased expression of GS caused by 2 months of intense ESLC was largely responsible for increasing muscle glycogen content following training in individuals with a SCI.

Citrate synthase (CS) is one of the enzymes in the Krebs Cycle. Its activity and protein expression is often utilized as a marker of the hallmark effect of endurance training, namely, increased muscle oxidative potential. It is well accepted that an increase in mitochondrial potential allows skeletal muscle to derive more ATP oxidatively from substrates other than glucose. Hence, a significant inverse relationship between CS activity and the rate of plasma glucose utilization during resting conditions is not a surprising finding (18). What is surprising is why endurance training leads to increased GLUT 4 protein content in skeletal muscle if the reliance on plasma glucose during exercise is decreased. The increased GLUT 4 content in the trained state allows for increases in maximal insulin-stimulated and contraction glucose uptake, but is not associated with increased basal metabolic glucose uptake rates. In fact, numerous studies have shown decreased glucose uptake rates during moderate exercise and resting conditions in the presence of increased total GLUT 4 skeletal muscle content in the trained state (82;112).

After 3 months of ESLC training, Mohr et al. (70) showed that CS activity doubled in the VL muscle of individuals with a SCI. The initial CS level of 12.3±1.4 mmol·kg d.w.$^{-1}$·min$^{-1}$. was in the range of what had previously been reported in sedentary able-bodied humans. The CS level of 24.8±2.2 mmol·kg d.w.$^{-1}$·min$^{-1}$. following training

in the individuals with a SCI was in accordance with previously reported data from studies in trained able-bodied individuals (70).

In a study by Kjaer et al. (60), muscle enzyme activity of not only HK, but also CS, plateaued after 3 months of training in individuals with a SCI, in which no further increases were observed with time despite continued training. These findings by Kjaer and colleagues (60) are in agreement with studies in able-bodied subjects showing early training induced adaptations in HK, CS actvity, and GLUT 4 content to endurance exercise that often plateau (77). Although upregulation of these proteins appears to occur co-temporally during training (70), some studies have shown HK and CS protein expression to not correlate directly with GLUT 4 expression (77). Phillips et al. (77) showed an increase in GLUT 4 expression without corresponding increases in HK II activity, while Houmard et al. (49) demonstrated a decrease in CS activity without a corresponding decrease in GLUT 4 content after training cessation in endurance trained subjects. In addition, the training induced adaptations of HK II, GS, and CS preceded changes in muscle fiber type composition of individuals with a SCI in both the study by Hjeltnes et al. (46) and the study by Kjaer et al. (60).

**TABLE 5:** *PROTEIN CHANGES ASSOCIATED WITH SCI AND ELECTRICALLY-STIMULATED TRAINING*

| Reference | N | Protocol | Duration | Content | | Activity | |
|---|---|---|---|---|---|---|---|
| | | | | GLUT 4 | HK | CS | HK |
| (60) | 10 | 30 min. 3x/wk | 6 months | NR | NR | NR | 150%↑ |
| (71) | 10 | 30 min. 3x/wk | 1 year | 105%↑ | NR | NR | NR |
| (16) | 5 | 30 min. 3x/wk | 8 wks | 72%↑ | NR | 56%↑ | NR |
| (46) | 5 | 30 min. 7x/wk | 8 wks | 378%↑ | 204%↑ | No Δ | 23%↑ |
| (70) | 10 | 30 min. 3x/wk | 3 months | NR | NR | 100%↑ | NR |
| (1) | 9 | NR | Chronic SCI | Comparable to controls | NR | NR | NR |

*NR, Not reported. All biopsies from VL. All subjects were complete SCI.*

## 1.7 STATEMENT OF PURPOSE

In skeletal muscle, glucose utilization is a function of three factors: glucose supply (arterial concentration times blood flow), glucose transport capacity of the sarcolemma, and intracellular glucose metabolism. Individuals with defects in any one of these three factors are predisposed to insulin resistance (16;24;39;86). The aim of the present study was to test the hypothesis that the morphological changes in skeletal muscle that occur as a result of SCI, and the associated alterations in the glucose transport system, can be partially or completely reversed by BWSTT. Based on several studies in which local muscle stimulation in persons with a SCI lead to improved glucose

homeostasis and enhanced insulin sensitivity, it was hypothesized that 6 months of BWSTT would result in the following changes in the subjects with a SCI:

1) Increase in area of all muscle fiber types in the VL muscle
2) Increase in GLUT 4 protein content in the VL muscle
3) Increase in CS activity and protein content in the VL muscle
4) Increase in capillary density and the number of capillaries per fiber in the VL muscle
5) Area under the time curve for plasma glucose would decrease during a modified 180 minute OGTT (100g)
6) Area under the time curve for plasma insulin would decrease during a modified 180 minute OGTT (100g)

# Chapter II

## Body-Weight Support Treadmill Training Induces Muscle Fiber Changes and Improves Glucose Tolerance in Individuals with an Incomplete SCI

## 2.1 Introduction

Traumatic spinal cord injury (SCI) results in the partial or complete loss of movement of skeletal muscle below the level of the spinal cord lesion. Inactivation and unloading of skeletal muscle compromises muscular performance, and in the past this has consequently rendered many individuals with a SCI to life in a wheelchair (79). The loss of physical mobility affects the metabolic state of the individual with a SCI, placing them at increased risk for inactivity-induced metabolic disease.

Profound muscle fiber atrophy, dramatic transformation to type II fibers, reduced capillary density, and a decrease in oxidative enzyme activity levels have been hypothesized as contributing factors to the increased fatiguability and reduced force generating capacity of skeletal muscle following a SCI (15). In addition, the muscular changes that occur following SCI are associated with impairments in glycemic regulation (17). In 1994, Bauman and Spungen (9) showed that 62% of individuals with quadriplegia and 50% of individuals with paraplegia had abnormal GT compared to only 18% of able-bodied controls. Increased incidence of non-insulin dependent diabetes mellitus and cardiovascular disease has been reported among individuals with a SCI (102) as their life-expectancy approaches that of able-bodied controls (40).

Rehabilitation in the SCI population progresses from passive stretching, weight-bearing and balancing exercises (33), to, in some instances, electrically-stimulated training (36). Although these forms of rehabilitation are associated with minor health benefits in the SCI population (54), they do very little to improve walking ability. However individuals with an incomplete SCI frequently identify independent walking as

one of their primary goals (22). Approximately 900 Canadians and ten thousand Americans sustain a SCI each year (22;94), and in more than half of these injuries, the individual retains some degree of motor control below the level of the injury (i.e., incomplete SCI) (6;101).

Studies have shown body-weight support treadmill training (BWSTT) to be a promising means of partially restoring ambulation in individuals with an incomplete SCI (27). Wernig and colleagues (108) studied two groups of subjects with SCIs who were matched for type of injury, time after injury and history of previous rehabilitation. The two groups were trained either conventionally (stretching, weight-bearing, etc.) or with the use of BWSTT for the same amount of time. Results showed that of eighteen subjects confined to a wheelchair, fourteen became independent walkers following BWSTT, compared to only one out of fourteen following conventional therapy.

Regular exercise training has proven to be an effective method for potentially preventing and treating impaired GT in the able-bodied population (8). In addition, two recent studies in individuals with a SCI have reported improved GT following periods of FES training (46;54). This appears to result from improved muscle morphology and post-exercise insulin sensitivity, and increased GLUT 4 content, enzyme activity, and muscle fiber capillary number (17;46). It might be expected that the weight-bearing and greater muscular involvement associated with BWSTT would have an even more profound effect on previously observed responses (17;46). Therefore, the purpose of this study was to investigate whether 6 months of BWSTT improved whole-body GT and some of the muscular parameters that are expected to influence glucose metabolism, in a group of individuals with an incomplete SCI.

It was hypothesized that BWSTT would induce an overall 'beneficial' adaptation in the muscle's metabolic profile that would be consistent with improved glycemic regulation and aerobic energy provision. Namely, that BWSTT would result in increases in muscle fiber area, the number of capillaries per fiber, GLUT 4 content, and CS content and activity.

## 2.2 Methods

### 2.2.1 Subjects

11 subjects, ten males and one female, were recruited to participate in the study. Two of the male subjects were unable to make the time commitment required to be in the exercise group, and therefore they served as controls. The average time since injury in the exercise group was 8.25 years (range 1.5-24). Following the time of their injury, a majority of the subjects were recreationally active (refer to Table 1), although no subject was involved in a formalized training program. All subjects were advised of the purposes of the study and associated risks, and all subjects gave written informed consent prior to participation. The project was approved by the Research Ethics Board of Hamilton Health Sciences, and conformed to guidelines involving use of human subjects as outlined in the Helsinki declaration on the use of human subjects in research.

**Table1**. *Subject Data*

| Subject | SCI Level | Date of Birth | Gender | Date of Injury | Years Post Injury | Prior Training |
|---|---|---|---|---|---|---|
| **1** | C5/6 | 17-Jun-68 | F | 6-Feb-86 | 16 | FES-legs |
| **2** | C5 | 2-Mar-77 | M | 2-Mar-77 | 24 | wheelchair basketball |
| **3** | T12/1 | 25-May-78 | M | 29-Oct-99 | 2 | basketball arm weights |
| **4** | C4 | 8-Oct-69 | M | 21-Nov-99 | 2 | none |
| **5** | T8 | 6-Feb-75 | M | 13-Jun-99 | 2 | arm cycle |
| **6** | C4 | 19-Feb-73 | M | 1-Oct-97 | 4 | Arm weights |
| **7** | C5 | 12-Oct-47 | M | 1-Jan-98 | 4 | Arm weights |
| **8** | C5/6 | 1-Jan-77 | M | 2-Jul-92 | 9 | none |
| **9** | C4/5/6 | 8-May-67 | M | 1-Jan-91 | 11 | none |

*All subjects had an ASIA score of C (motor function < 3 grade is preserved below the lesion level). Subjects did not participate in another training program during the study.*

### 2.2.2 Design

A longitudinal pre-post design was used in which the subjects performed an OGTT and had a leg biopsy before and after 67 walking exercise sessions, spanning approximately 6 months on the Body-Weight Support Treadmill (BWST).

### 2.2.3 Exercise Protocol

Upright walking exercise was completed on a motor driven treadmill (Woodway USA Inc., Foster, CT) while a harness suspended from an overhead pulley system, supported the subject's body weight. Initial body weight support (BWS) and speed of the treadmill were chosen according to the individual abilities of each of the subjects. Most

subjects began training with 60% or more of their body weight supported, and walked at treadmill speeds of less than 0.6 km/h. Depending on the amount of assistance required by each subject, it was usual to have one therapist on either side of the subject to aid in leg movement. With respect to leg movement, assistance was particularly important in bending the knee and hip joint during the swing phase of the gait pattern to ensure full knee extension and stabilization during the stance phase of the gait cycle. Subjects were instructed to place their body weight over their fully extended leg during the stance phase of the walking cycle. If required, a third therapist was stationed behind the subject to assist in weight transfer from one leg to the next. Treadmill speed, duration on the treadmill, and the amount of body weight supported by the subject's own legs were increased according to their own rate of improvement. After a number of sessions, many subjects were able to walk without the aid of therapists while supporting a large majority, if not all, of their body weight. The usual training time was 30-40 minutes per session, for 3 sessions per week. For each walking bout, the duration on the treadmill, the speed of the treadmill, and the amount of body-weight support given to the subject, was recorded.

### 2.2.4 Muscle Biopsy

Needle biopsy samples (5mm Bergstrom needle) were obtained from each subject under local anesthesia (2% lidocaine) using manual suction. The biopsies were taken from the middle portion of the vastus lateralis on the same leg before and after the BWSTT. Fat and connective tissue was immediately removed from each biopsy sample,

which was then dissected into three portions. Two of the portions were placed in 1.5ml eppendorf tubes and frozen in liquid nitrogen. The third portion was placed in optimum cutting temperature (OCT, Tissue Tech™) embedding medium with its fibers perpendicular to the plane in which it was to be cut. The muscle portion placed in OCT was then quick frozen in isopentane cooled by liquid nitrogen, and like the other two muscle portions, was stored at –70 °C until subsequent analysis.

### 2.2.5 Fiber-Type Analysis: Histochemistry

Before performing the ATPase stain, a Hematoxylin and Eosin (H+E) stain was performed on 10μm sections of muscle from each subject to ensure the fibers were oriented correctly in the embedding medium. The ATPase stain was used to identify the fiber type of the VL muscle fibers studied. 8μm serial tissue sections of muscle were cut by a cryostat (model MICROM HM500OM, Walldorf, Germany) and transferred to microscope slides. The slides were incubated in 50ml of acid preincubation solution at pH 4.60, followed by a rinse and a second incubation in a 100ml solution containing 0.003M ATP (SIGMA A2383, St. Louis, MO, USA) and 0.059M calcium chloride (BDH 10070) at a pH of 9.4. The second incubation was completed at 37°C in a temperature-controlled shaker (624 Environmental, New Brunswick Scientific Co., Edison, NJ, USA). After 45 minutes the tissue section was removed from the ATP solution and placed in a 2% cobalt chloride ($CoCl_2$) solution. The tissue was then placed in a solution of 1% ammonium sulphide [$(NH_4)_2S$] for 1 minute. The ATPase enzyme present in various muscle fiber types is dependent upon the influence of pH. By preincubating the tissue

sections at a pH of 4.60, the ability of the myofibrillar ATPase enzyme to split ATP into ADP and Pi in fast twitch muscle fibers (Type IIa and Type IIx) is selectively blocked, or at least attenuated. Therefore at pH 4.60, Type I fibers stain dark, Type IIa fibers stain light, and Type IIx fibers stain medium (because they contain a faster ATPase enzyme than Type IIa).

Once the muscle tissue was properly stained, pictures of the stain were collected under 200x magnification with the use of a microscope (Olympus BX60, Melville, NY) and camera (SPOT, Diagnostic Instruments Inc., Houston, TX, USA). Images were analysed for fiber number, size and area by using the Image-Pro Plus™ (V4, Media Cybernetics, Des Moines, Iowa, USA) computer program. Muscle fibers were classified as type I, IIa, IIax or IIx based on visual selection of staining intensity. If the stain was not clear enough to easily distinguish the different fiber types, then optical density (OD) measurements were also used as an aid in grouping fiber types. The four histochemically delineated fiber type areas were subsequently collapsed into the three major fiber types (I, IIa, and IIx) using the formulae: IIa + ½ IIax = IIa, and IIx + ½ IIax = IIx.

### 2.2.6 Western Blotting Protocol

Depending on the weight of the muscle sample used, a corresponding amount of 0.5% SDS buffered saline (10mM Tris base; 5mM EGTA; 0.1mM DTT; 2mM PMSF) was added to the muscle during the homogenizing process. The same homogenization buffer was used for both GLUT 4 and CS protein content. The Bradford protein assay (#500-0006, Bio-Rad, Hercules, CA) was completed on each subject's homogenate for

protein content determination. The Bradford protein assay was completed to ensure that equal amounts of protein were loaded in each lane during electrophoresis. All samples were run in triplicate and the coefficient of variation was less than 5% for each sample. Crude muscle homogenate was stored in aliquots at -70°C until analysis. Proteins were separated using a 4% SDS-polyacrylamide stacking gel and either a 12% SDS-polyacrylamide separating gel for CS or a 10% SDS-polyacrylamide gel for GLUT 4. Prior to electrophoresis, each homogenate was mixed with a buffer containing 10% sodium dodecyl sulfate (SDS) and 2.5% beta-mercaptoethanol. Pre-training and post-training samples for each subject were loaded in adjacent lanes beside on the same gel, along with a broad range molecular weight standard (#161-0319, Bio-Rad). The gels were run with the power supply set at 100V for 1 hr at room temperature. After electrophoresis, the proteins were transferred to a synthetic PVDF membrane (Bio-Rad, #162-0184). Following transfer to the synthetic membrane, the protocols for GLUT 4 and CS were as follows:

**GLUT 4:** The PVDF membrane was placed in a 3% Gelatin solution (Sigma, #G-7765) dissolved in Tween-Tris-buffered saline (TTBS; 500mM NaCl, 20mM Tris, HCl, pH 7.5) with 0.1% Tween (Bio-Rad, #170-6531) to block for 1 hour at 4°C. This was followed with a 3x7 minute wash in TTBS, and a second block in 5% skim milk powder dissolved in TTBS. The second block was followed again by a 3x7 minute wash in TTBS before applying the 1° antibody. The rabbit anti-human GLUT 4, 1° antibody (H-61, polyclonal IgG, # sc-7938, Santa Cruz Biotechnologies, Santa Cruz, CA) was then incubated onto the membrane at a 1:500 dilution (in TTBS) for 2 hours at room temperature. This was followed by a 4x10 minute wash in TTBS, and the application of

the 2° antibody (Bio-Rad, Goat Anti-Rabbit IgG (H+L)-AP conjugate). The goat anti-rabbit 2° antibody and a biotinylated-streptavidin alkaline phosphatase enzyme (Bio-Rad) were incubated onto the membrane at a 1:3000 dilution (in TTBS) for 1 hour at room temperature. The membrane was then washed before being exposed to immunostar substrate (Bio-Rad) and Kodak film, which was subsequently developed, digitized using the Epi-Illumination UV Darkroom™, and analysed for band density using a computer program from Labworks™.

**CS:** The membrane was placed in a 5% milk solution to block overnight. This was followed by a 3x7 minute wash of the membrane in TTBS. The 1° antibody (rabbit polyclonal IgG; gift of Dr. B.H. Robinson) was then incubated onto the membrane at a 1:1000 dilution (in TTBS) for 1 hour at room temperature. This was followed by a 3x7 minute wash in TTBS and the incubation of the 2° antibody (Bio-Rad Goat Anti-Rabbit IgG (H+L)-AP conjugate) onto the membrane for 1 hour at room temperature. The membrane was given a final 3x7 minute wash before it was developed onto Kodak film and analysed using densitometry.

### 2.2.7 Citrate Synthase Activity Assay

This reaction involves the measurement of citrate synthase activity by linking the release of CoASH to the colormetric agent DTNB (5,5-dithiobis-2-nitrobenzoate). After the muscle sample was weighed, 425µl of prepared homogenizing buffer was added to the muscle sample. One ml of Tris buffer (0.1M, pH 8.0) heated to 37°C was added to a cuvette containing 10µl of DTNB (4mg/ml of Tris buffer) and 2µl of acetyl CoA (30mM

in $H_2O$). To this, 10µl of muscle homogenate was added and mixed. The spectrophotometer (Shimadzu UV-1201, Kyoto, Japan) was zeroed and 10µl of Oxalo-acetic-acid (6.6 mg/ml Tris buffer) was added to start the reaction. After a short lag time, the enzyme reached its maximal velocity and the change in absorbance was linear. During the linear period of steady activity, the rate of change in absorbance/time was used in the calculations. Absorbances were taken at 412nm, at 60s for 2 minutes. The activity was calculated and expressed as µmol· $min^{-1}$· g $w.w.^{-1}$. The intra-assay CV was less than 9.0%.

### 2.2.8 Capillary Stain: Amylase Periodic Acid Shiff

10µm sections of muscle were cut with the cryostat (MICROM, HM500OM, Walldorf, Germany) and placed on slides. The tissue sections were incubated at 37°C in a 1% α-amylase solution (Sigma A6255) for 40 minutes. Following rinsing, the tissue sections were then placed in a periodic acid solution (Sigma P5463) for 12 minutes at room temperature. After another rinse, the tissue sections were incubated at 37°C in Schiff's solution (Pararosaniline, Sigma P7632; Sodium metabisulfite, Sigma S1516; Activated charcoal, Sigma C4386) for 10 minutes.

Once the muscle tissue was properly stained, pictures of the stain were collected under 200x magnification with the use of a microscope (Olympus BX60) and camera (Diagnostic Instruments SPOT). Images were analysed for total fiber area, fiber number, and capillary number by using the Image Pro-Plus™ computer program. For comparative purposes between pre and post training, we examined the capillary density (capillaries

per square millimeter of cross sectional fiber area), and the capillary-to-fiber ratio (C/F) from a transverse section of muscle.

### 2.2.9 Oral Glucose Tolerance Test (OGTT)

All of the subjects reported to the testing centre in an overnight fasted condition. A catheter was inserted into an antecubital forearm vein for withdrawing the subject's blood throughout the test, and was kept patent using sterile 0.9% saline solution. During each of the tests, the subjects ingested 1,000ml of room temperature tap water containing 100g of glucose. The drink was given in four equal volumes (25g of glucose in 250ml of water), separated by a half hour, over a 90 minute period (0, 30, 60, 90 min.). Baseline blood samples were taken from the subject, followed by ingestion of the first 250ml drink. Venous blood samples were collected into heparinized evacuated tubes (Vacutainer ™) at the following times: 0 min, 10 min, 20 min, and then every 20 minutes for the remainder of the 3 hour test. A schematic representation of the study protocol is shown in Figure 1. Whole blood from the Vacutainer™ was then placed into an eppendorf tube and analysed immediately by an automatic glucose analyzer (YSI 2300 STAT plus, Yellow Springs, Ohio, USA). The remainder of the blood in the Vacutainer™ was spun in a centrifuge (International Equipment Company 21000R, Needham Heights, MA, USA) at 4500 RPM and 4 degrees Celcius for 10 minutes. After centrifugation, the supernatant (plasma) was transferred to eppendorf tubes and stored at –80 degrees Celcius until the insulin radioimmunoassay was performed.

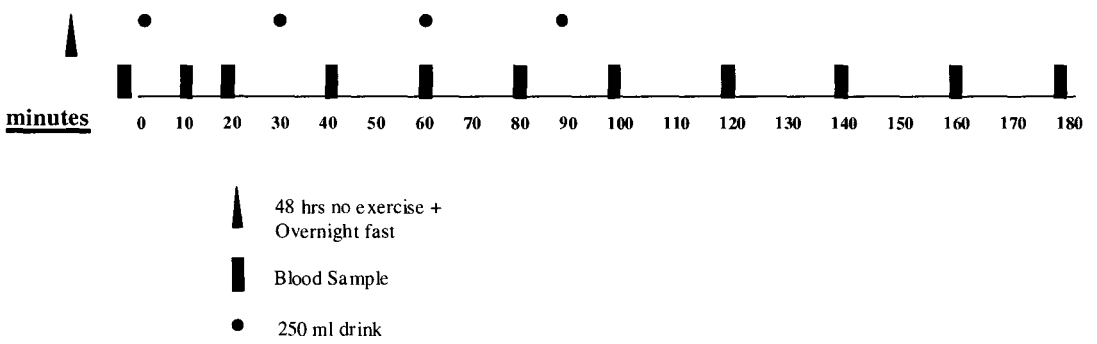



Figure 1. Oral Glucose Tolerance Test Protocol

### 2.2.9.1 *Insulin radioimmunoassay*

Insulin antibody-coated tubes (Coat-A-Count Coated Tube RIA Kit) were purchased from Diagnostic Products Corporation (Los Angeles, CA) for this assay. 200µl of plasma from each subject was transferred to a single Insulin Ab-coated tube. Each subject was run in duplicate. 1.0ml of $^{125}$I Insulin was added to every tube. $^{125}$I-labelled insulin competes with insulin in the patient sample for sites on the insulin-specific antibody coating the side of each tube. The tubes were left to incubate for 24 hours at room temperature. Following incubation, all visible moisture was removed from

the tubes. Decanting each tube isolates the antibody-bound fraction of the radiolabelled insulin. The tubes were then counted in a gamma counter (Packard MINAXI Auto Gamma 5000 series, Downer's Grove, IL, USA) to yield a number, which by way of a calibration curve, converts to a measure of the insulin present in each subject's sample.

### 2.2.10 Statistical Analysis

Blood glucose and plasma insulin data during the OGTT were analyzed using a two-factor repeated measures analysis of variance (ANOVA), with treatment (pre/post exercise training) and time (minutes) as the within factors. Significant main effects and interactions were further analyzed using a Tukey post hoc test. In addition, a paired t-test was used to analyze whether AUC for blood glucose and plasma insulin was significantly different following BWSTT. For all other data, paired t-tests were run on the pre and post-training results. Correlations were run using Pearson-product correlation analyses on all relevant data. Statistical significance for all analyses was accepted as $P \leq 0.05$. All data in the text and figures are presented as means $\pm$ SE.

## 2.3 Results

### 2.3.1 Histochemistry Data Analysis

Post training values for type I and type IIa fiber size showed significant increases of 1,653 $\mu m^2$ (P=0.01; figure 6) and 1,402.5 $\mu m^2$ (P=0.05) respectively, in comparison to pre training values. Type IIx fiber size increased by 1,038 $\mu m^2$, although this was not significant (P=0.06). Average fiber size pre training for all 3 fiber types combined was 3,718±357 $\mu m^2$, and after training average fiber size significantly increased to 5083±303 $\mu m^2$ (P<0.001).

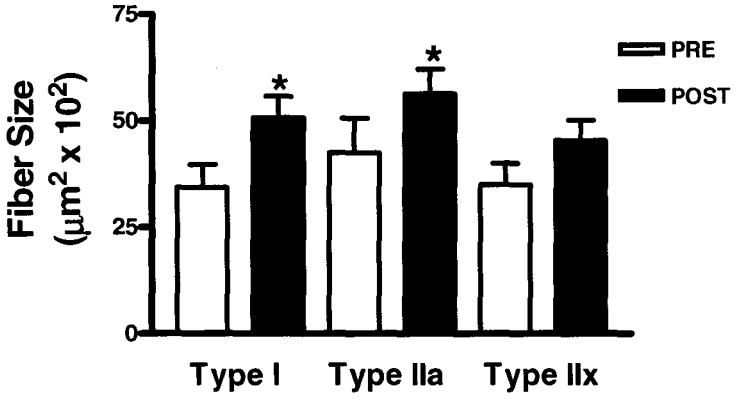



Figure 2. Fiber size (x$10^2$) before and after BWSTT for type I, type IIa, and type IIx fibers.
* Significantly different (P<0.05) from pre BWSTT. Values are means±SE.

Type IIa fiber % distribution significantly increased from 27±5% before training to 42±7% after training (P=0.02; figure 7). Post training values for type I, type IIax, and type IIx fiber % distribution remained unchanged (P=0.18, P=0.30, and P=0.09, respectively) in comparison to pre training values.

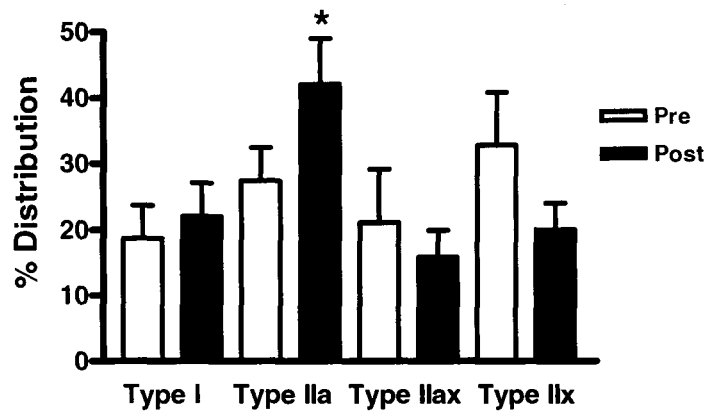



Figure 3. % Distribution before and after BWSTT for type I, type IIa, type IIax, and type IIx fibers. * Significantly different (P<0.05) from pre BWSTT. Values are means±SE.

Type IIa % area values significantly increased (P=0.03; figure 8) from 41±5% pre training to 53±5% post training. Type IIx % area values significantly decreased (P=0.009) from 42±5% pre training to 25±2% post training. Type I % area did not change (4±2%, P=0.07) with training.

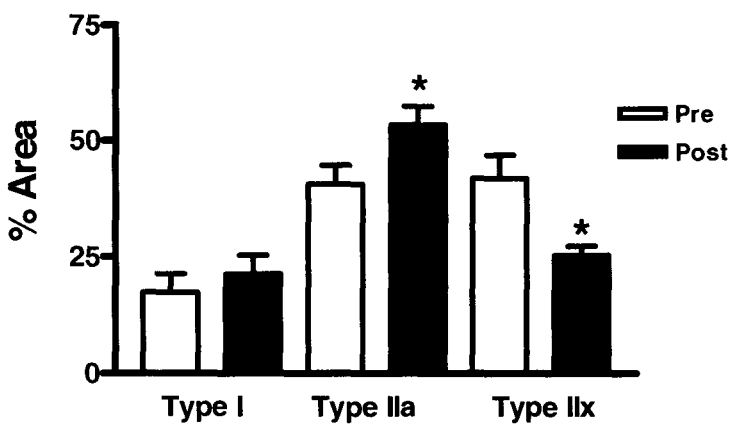



Figure 4. % Area before and after BWSTT for type I, type IIa, and type IIx fibers. * Significantly different (P<0.05) from pre BWSTT. Values are means±SE.

### 2.3.2 Protein Data Analysis

Post training GLUT 4 protein content significantly increased by 127±43% (P=0.002; figure 9). Post training CS protein content significantly increased by 170±49% (P=0.003; figure 10) in comparison to pre training values. Post training CS activity values significantly increased by 24±10% (P=0.01; figure 11), from 6.9±1 $\mu mol \cdot min^{-1} \cdot g$ w.w.$^{-1}$ pre training to 8.4±1 $\mu mol \cdot min^{-1} \cdot g$ w.w.$^{-1}$ post training. A significant correlation existed for the % change in GLUT 4 content and the % change in CS activity for all subjects following training (R=0.864; P=0.006).

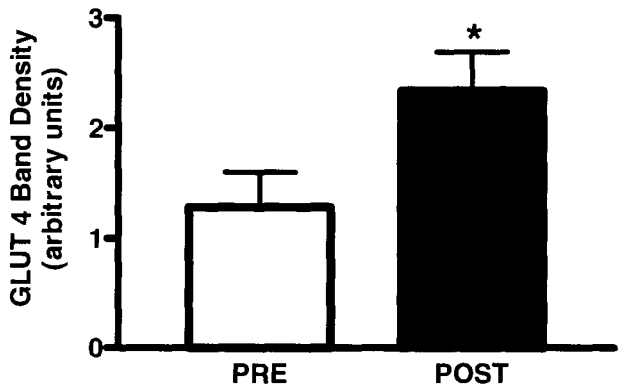



Figure 5. Densitometry on GLUT 4 blot (arbitrary units x $10^2$) before and after BWSTT. * Significantly different (P<0.05) from pre BWSTT. Values are means±SE.

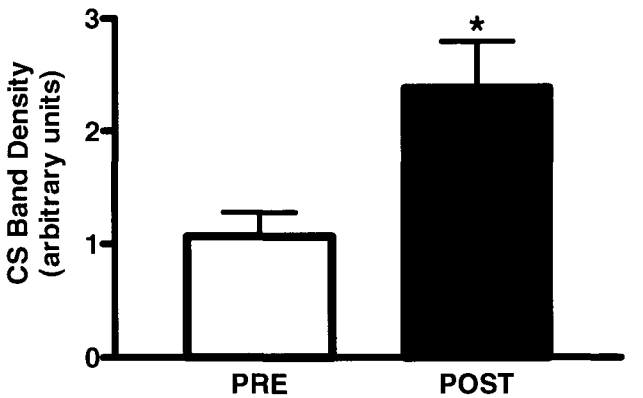



Figure 6. Densitometry on CS blot (arbitrary units x $10^2$) before and after BWSTT. * Significantly different ($P<0.05$) from pre BWSTT. Values are means±SE.

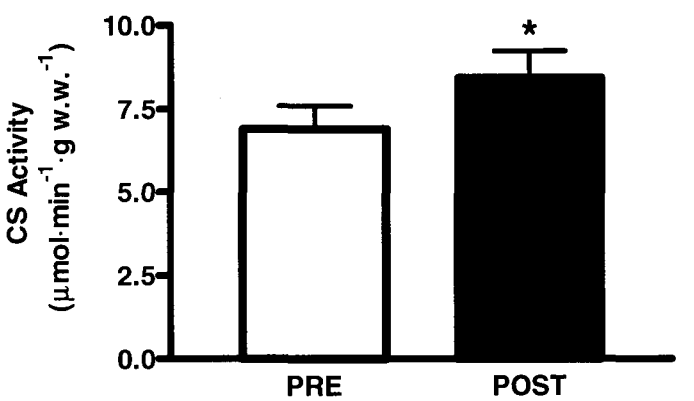



Figure 7. CS activity before and after BWSTT. * Significantly different ($P<0.05$) from pre BWSTT. Values are means±SE.

### 2.3.3 Capillarization Data Analysis

The number of capillaries per fiber post training was 0.72±0.06, which showed no change in comparison to the pre training value of 0.73±0.05 (figure 12). Post training values for capillary density significantly decreased by 35±10% (P=0.02; figure 13), from 188±36 capillaries·mm$^{-2}$ fiber area pre training to 115±24 capillaries·mm$^{-2}$ fiber area post training.

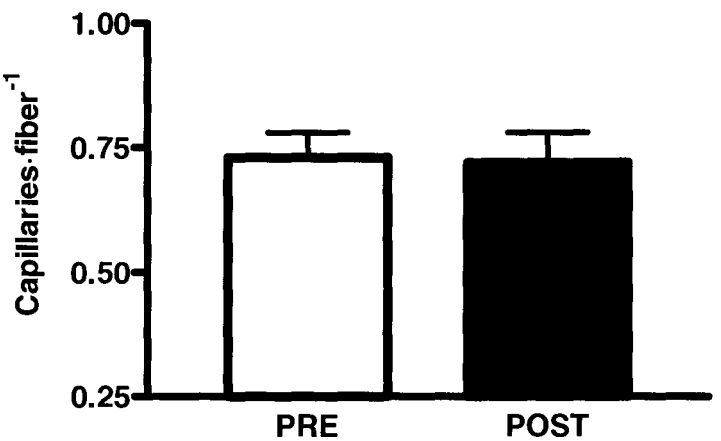



Figure 8. The number of capillaries per fiber before and after BWSTT. Values are means±SE.

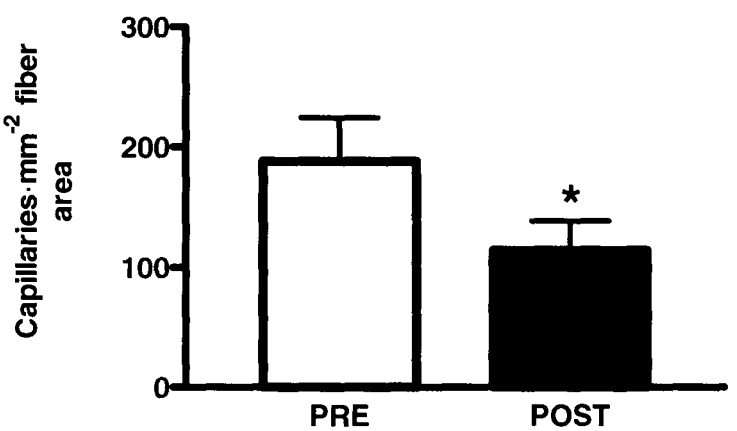



Figure 9. Capillary density before and after BWSTT. * Significantly different (P<0.05) from pre BWSTT. Values are means±SE.

### 2.3.4 OGTT Blood Glucose

There was a significant interaction of time and training during the OGTT, where blood glucose concentration was significantly lower at 140 minutes of the OGTT after BWSTT ($P<0.05$; figure 2). Blood glucose area under the time curve decreased 8±2% ($P<0.01$; figure 3) following BWSTT.

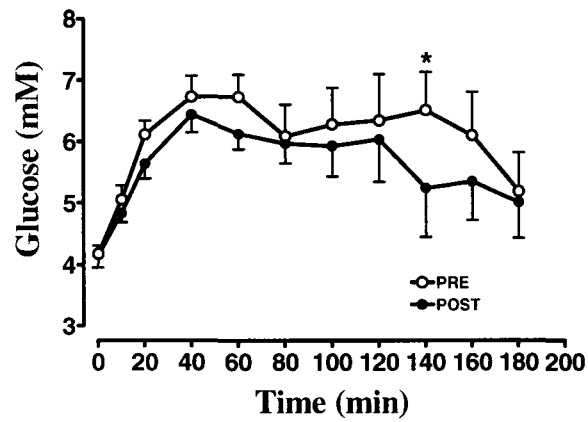



Figure 10. Blood glucose concentration pre and post BWSTT. * Significantly different ($P<0.05$) from post BWSTT. Values are means±SE.

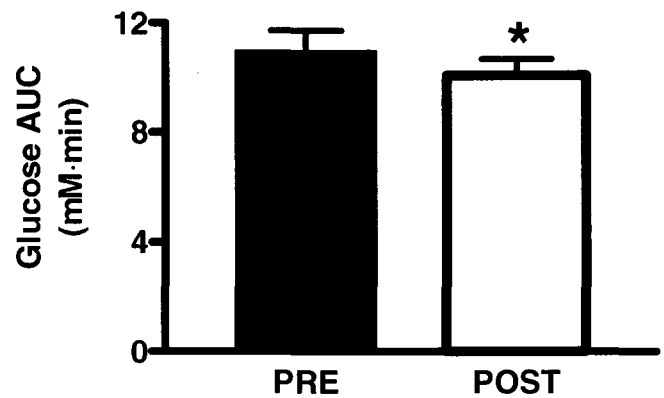



Figure 11. Area under the time curve ($x10^2$) for blood glucose during the OGTT, before and after BWSTT. * Significantly different ($P<0.05$) from pre BWSTT. Values are means±SE.

### 2.3.5 OGTT Plasma Insulin

Plasma insulin was increased as a result of the OGTT and remained unaffected by training (P=0.06 training main effect, P=0.43 interaction; figure 4). Plasma insulin area under the time curve significantly decreased 20±11% (P=0.04; figure 5) after training in comparison to pre training values. The Insulin Resistance Index (glucose AUC x plasma AUC) significantly decreased by 27±11% (P=0.04).

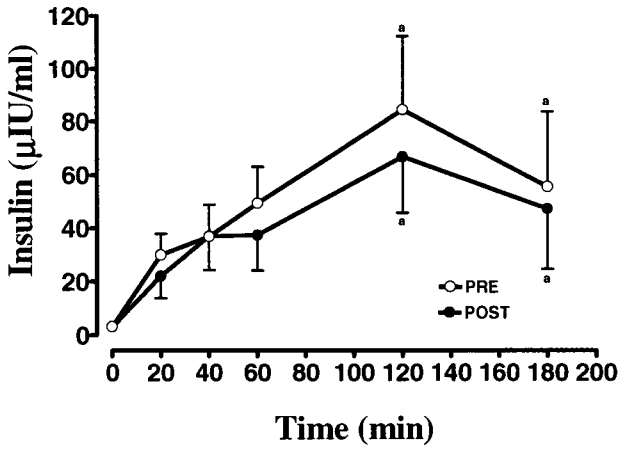



Figure 12. Plasma insulin concentration before and after BWSTT. Means with the same letter (a) are significantly different versus 0 (P<0.05). Values are means±SE.

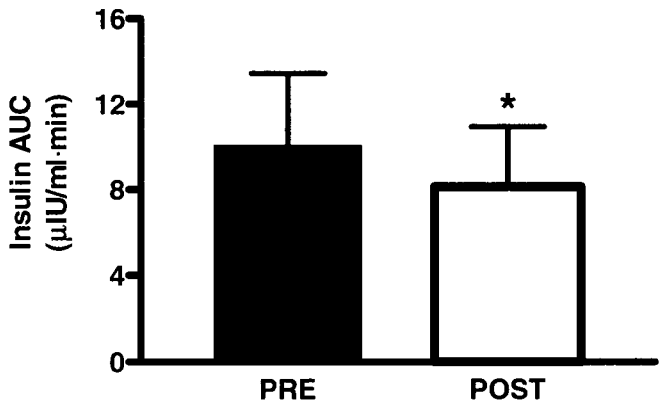



Figure 13. Area under the time curve for plasma insulin ($x10^4$) during the OGTT, before and after BWSTT. * Significantly different (P<0.05) from pre BWSTT. Values are means±SE.

### 2.3.6 Subject Performance Data Analysis

Treadmill walking speed significantly increased by 190±59% ($P<0.001$; figure 14), from an average of 0.54±0.11 km/hr during the first 3 weeks of training to 1.19±0.11 km/hr at 6 months into training. Duration on the treadmill significantly increased by 81±23% ($P=0.001$; figure 15), from 23±4 minutes during the first 3 weeks of training, to 35±3 minutes at 6 months into training. The % of body-weight support that the subjects required while walking on the treadmill significantly decreased by 65±8% ($P<0.001$; figure 16), from 65±5% during the first 3 weeks of training to 24±5% at 6 months into training. According to the modified Wernig Scale (McMaster University), 3 of the 8 subjects (S3, S5, S7) improved their functional classification of locomotor capability.

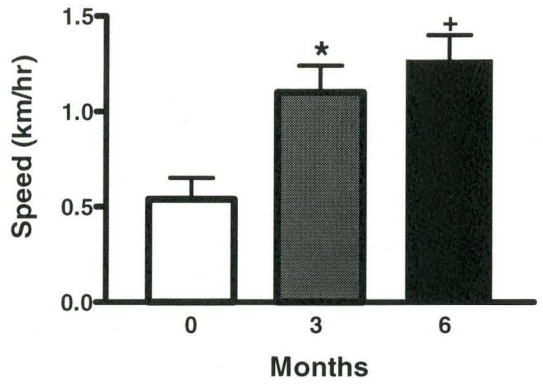



Figure 14. Treadmill walking speed at 0, 3, and 6 months of training. * Significantly different (P<0.05) from walking speed at 0 months. + Significantly different (P<0.05) from 3 months and 0 months. Values are means±SE.

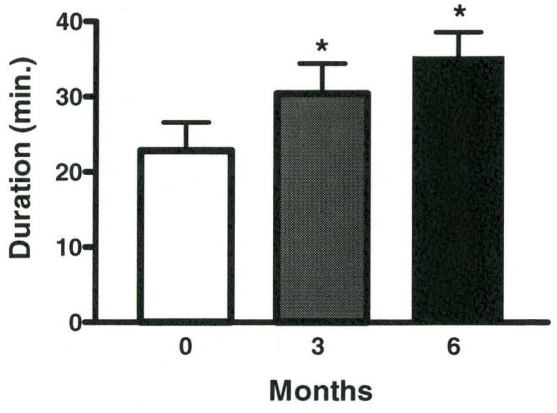



Figure 15. Walking duration on the treadmill for a single exercise session at 0, 3, and 6 months of training. * Significantly different (P<0.05) from duration at 0 months. Values are means±SE.

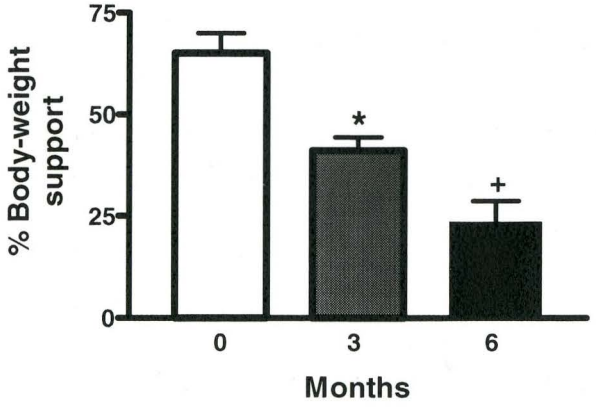



Figure 16. Percent body-weight support supplied by the harness during treadmill walking at 0, 3, and 6 months of training. Significantly different (P<0.05) from BWS at 0 months. + Significantly different (P<0.05) from 3 months and 0 months. Values are means±SE.

**Table 2.** *Modified Wernig Scale Results*

| Subject | Pre-Test | 6 month Test |
|---|---|---|
| S1 | 4 | 4 |
| S2 | 7 | 7 |
| S3 | 0 | 4 |
| S4 | 8 | 7 |
| S5 | 0 | 7 |
| S6 | 0 | 0 |
| S7 | 0 | 2 |
| S8 | 0 | 0 |
| S9 | 0 | 0 |

## 2.4 Discussion

After ingestion of an oral glucose load, 65-90% of total glucose is utilized or stored as glycogen by skeletal muscle, whereas adipose tissue is responsible for the disposal of less than 10% of orally administered glucose (72). Consequently, glucose tolerance, as measured by an OGTT, is partly a reflection of the skeletal muscle/adipose tissue mass ratio (24). Our results suggest that BWSTT may help improve GT in individuals with a SCI by increasing the size of their muscle fibers. It is well documented that muscle hypertrophy is maximized by chronic exposure to work loads requiring high-tension development (e.g., resistance training) (67). Although walking exercise in the able-bodied population does not generally produce muscle tension large enough to induce fiber size enlargement, walking for individuals with a SCI, even with the aid of body-weight support, may act as a relative resistive stimulus as well as being aerobic in nature.

Due to a lack of strength, gait in individuals with a SCI is often characterized by an inability to properly straighten the knee joint during heel strike and stance (6). Following every heel plant, the subjects would be required to flex their VL muscle, enabling them to push off of the treadmill and properly straighten their leg in preparation for the next heel plant. Our subjects displayed significant increases in the sizes of their type I and type IIa muscle fibers, and every subject, except for one, displayed an increase in type IIx fiber size following BWSTT. This hypertrophy may have allowed greater tension development resulting in improved ability to support their weight following heel plant. However, the direct impact of the increase in muscle fiber size on gait and walking performance was not assessed.

The myosin ATPase stain revealed the existence of both pure and "hybrid" fiber types in our subjects before and after training. Pure fiber types contain a single MHC isoform, whereas hybrid fibers contain two or more MHC isoforms (76). A large amount (21±8%) of fibers were classified as type IIax hybrid fibers in the subjects with a SCI studied. The significance of multiple MHC isoforms in single muscle fibers is still not clear, although it is believed that hybrid fibers are representative of a muscle undergoing transition or new functional demands (5), which usually is the case following SCI. Studies have shown a reduction in hybrid single muscle fiber distribution in young and elderly individuals following resistance training (110), which has led some researchers to speculate that hybrid fibers have a greater adaptive potential, making them more suitable for fiber type transition when met with altered functional demands (5). Transitions in MHC isoform expression follow a sequential order from type IIx → type IIax → type IIa following training (75), and the fact that our subjects showed a significant increase in the

% distribution of their type IIa fibers following training, support this theory of hybrid fibers being highly adaptive. However, no significant change in the % distribution of the hybrid fibers themselves, were found in our subjects following training. This suggests that the pre training type IIx fibers, which would have been responsible for transition to the type IIax fibers, are as adaptable as the hybrid fibers.

Prior to training, the individuals with a SCI who participated in our study, had on average, a VL muscle composed of over 40% type IIx fibers. The effects of SCI on muscle fiber transformation are obvious when one considers that most studies find the VL muscle in able-bodied individuals to be composed of anywhere from 10-20% type IIx fibers (5;97). Phenotypically, the long-term effect of SCI is a large increase in the amount of type II fibers (2), and a corresponding decrease, or complete disappearance of type I fibers (1;2). According to Andersen et al. (2), MHCI seems to be the isoform most dependent on neural input. The individuals with a SCI who participated in our study were on average, 8.5 years post injury, long enough for a substantial fiber type transition to have occurred. Our subjects had a VL composed of 17% type I fibers. This is a much smaller proportion than has previously been reported in able-bodied subjects who generally average between 40-50% Type I fibers (97). The proportion of type I fibers in the current subjects is higher than what has previously been reported in individuals with a complete long standing SCI (range 0-4%) (1;2;70). With partial innervation to the muscle, and consequently the ability to maintain some movement below the level of the spinal cord lesion, one would hypothesize that individuals with an incomplete SCI maintain a larger proportion of type I fibers in comparison to individuals who have suffered from complete lesions.

Fiber type conversion in the SCI population has been demonstrated following electrical stimulation (ES) training (2;70) but has been primarily limited to shifts within the fast fiber population from type IIx to type IIa fibers. In our subjects, type IIx and type IIa fiber proportion decreased and increased respectively, and % type I fiber area remained the same. One year of electrically-stimulated leg cycling (ESLC) in subjects with a complete SCI induced a transformation in VL muscle fiber type, from a muscle expressing both MHCIIx and MHCIIa fibers, to a muscle dominated by fibers expressing exclusively MHCIIa (2). In comparison to studies that have utilized ES training (2;70), the use of BWSTT in our study produced a much smaller reduction in the proportion of type IIx fibers in the VL muscle. This is likely due to the much greater potential for change in persons with complete SCI, who undergo much greater shifts toward type IIx fibers following injury.

The stimuli for synthesis of GLUT 4 in our subjects was most likely a local contraction-dependent phenomenon, since ES contraction induces increases in GLUT 4 content, but does not involve centrally-driven neural input (16). Mohr et al. (71) found a 105% increase in GLUT 4 content following 1 year of ESLC exercise in complete individuals with a SCI. Our results reveal a similar increase in GLUT 4 content following only 6 months of BWSTT in incomplete individuals with a SCI. By using a more intense training protocol involving 7 ESLC sessions/wk, as opposed to the protocol of 3x/wk used in our study and the study by Mohr et al. (71), Hjeltnes et al. (46) found even larger increases in GLUT 4 expression in the VL muscle of subjects with a SCI.

Studies utilizing ES training have found GLUT 4 content in the VL muscle and whole-body insulin sensitivity in subjects with a SCI do not vary in parallel (71).

Changes in GLUT 4 content do not necessarily correlate with whole-body glucose tolerance because adaptations to GLUT 4 expression tend to be localized to the individual muscles undergoing the contractile activity during training (25). More to the point, Daugaard and Richter (21) found GLUT 4 was increased only in the fibers that were recruited during exercise. Hence, it makes sense that the greatest increases in GLUT 4 content will occur in human muscles that are stimulated or voluntarily activated to result in recruitment of all three major fiber types during training. The increase in GLUT 4 content and hypertrophy of type I, IIa, and IIx fibers demonstrated in our subjects indicate that BWSTT likely caused the recruitment, to some extent, of all three major fiber types. In comparison to electrically stimulated training, we believe that the greater muscular involvement associated with BWSTT, caused multiple fiber types from muscles aside from the VL (which was the only muscle we examined) to beneficially change their metabolic parameters affecting glucose tolerance, such as GLUT 4 expression. Cortez et al. (19) demonstrated that increasing the amount of active muscle during exercise causes further increases in glucose uptake during exercise. Therefore, by utilizing BWSTT as a mode of exercise, it appears that individuals with a SCI are able to stress a large enough proportion of their body's total muscle mass to produce metabolic changes that directly and beneficially affect whole-body glucose tolerance.

In rodents, GLUT 4 content has been found to be greater in oxidative muscles than glycolytic muscles (37). This indicates that an increase in oxidative capacity seen during the training period in our study would be expected given the increased GLUT 4 content. However, not all studies in humans have found a correlation between fiber type proportion and GLUT 4 content in muscle (3;20). Despite an almost complete lack of

type I fibers and a large proportion of type IIx fibers, Aksnes et al. (1) found that GLUT 4 content in the VL muscle of quadriplegic patients was comparable to healthy control values. According to more recent research, it appears that GLUT 4 content may correlate more closely with the oxidative capacity of a muscle, than to its fiber type composition (21). In our study, changes in GLUT 4 content correlated with changes in CS activity, and not changes in type IIa fiber proportion among the trained subjects. Like CS activity, changes in fiber type distribution tend to be representative of a muscle's overall activity level. However, training-induced increases in mitochondrial enzymes tend to precede transitions in MHC content within muscle due to the much higher rate of turnover in myoplasmic versus myofibrillar proteins (46;60;77). Consequently, studies have shown a wide range in metabolic enzyme activities within and between histochemically determined fiber types (74). Therefore, although the adaptation in CS activity and content at 6 months may have plateaued (60), we speculate that MHC transitions were still occurring after 12 months of training. Had we given our subjects a longer training protocol (e.g., 1 year), we may have witnessed a correlation between the change in GLUT 4 content and the change in fiber type distribution, in addition to oxidative enzyme activity.

After 6 months, no change occurred in the number of capillaries per fiber in the individuals undergoing BWSTT. Due to the profound changes in morphological and metabolic parameters we found in the VL muscle following training, we had hypothesized that BWSTT might provide an adequate stimulus to induce increases in muscle capillarization. At 3 sessions per week, 6 months of BWSTT was potentially too short in duration to witness the effects of a training-induced angiogenesis.

Three previous studies involving ES training have examined capillarization in persons with a SCI (17;67;84). Of the three, only one found increases in capillary number with training (17). The training-induced increase in capillarization resulted in a capillary number still well below that of able-bodied controls, which is generally reported to be 1.5 capillaries per fiber (17). Before training, our subjects had a capillary per fiber ratio of 0.73, demonstrating a dramatic reduction in capillary number that occurs in the SCI population (1). An increase in the number of capillaries following exercise training facilitates increased insulin and glucose transport to the muscle by diminishing diffusion distance (43). Any benefits from an increase in capillary number in the Chilibeck et al. study (17), were offset by a proportional increase in fiber area within their subjects following training. Capillary density did not significantly change, and therefore diffusion distances for glucose and insulin from the capillary to the muscle would have remained relatively the same. Due to the combination of no angiogenesis and fiber size enlargement, the significant decrease in capillary density witnessed in the subjects in our study, may have affected the supply of oxygen and substrate to their muscles. However, if this was the case, it did not affect their performance on the treadmill, which increased dramatically for all three measures (speed, duration, % BWS) in our study.

The results from the present investigation indicate that 6 months of BWSTT in persons with an incomplete SCI resulted in improved glucose tolerance, as represented by attenuated blood glucose and plasma insulin concentrations following training. Plasma insulin area under the time curve (AUC) significantly decreased in our subjects as a result of BWSTT. Despite the large decrease in plasma insulin concentration during the post training OGTT, blood glucose AUC also showed a significant decrease, indicating a

training–induced increase in insulin sensitivity occurred in our subjects. According to our findings, we can speculate that the increase in insulin sensitivity was a result of the increase in muscle GLUT 4 content in the exercised muscles, possibly in combination with an increase in leg muscle mass, as indicated by an increased muscle fiber area.

A number of investigators have shown improvements following varying periods of BWSTT (31;108). In one study, twenty of the twenty-five patients who were initially wheelchair bound became independent walkers (109). Previous investigators have credited the improvement in walking capability in the SCI population to the enhancement of specific neural circuitry within the spinal cord (78) and the more efficient use of remaining muscle function (79) following BWSTT. Our study is the first to show increases in treadmill ambulation in concordance with improved muscle size and metabolic parameters.

## 2.5 Conclusion

Skeletal muscle is the primary site of glucose disposal in the body, and consequently plays a predominant role in the regulation of blood glucose levels after the ingestion of a meal. Following SCI, skeletal muscles below the level of an upper motor neuron lesion undergo marked changes in muscular properties that affect glucose and exercise tolerance. Our results are the first in this field to provide evidence that BWSTT leads to beneficial morphological and metabolic changes in skeletal muscle fibers that improve glucose tolerance and exercise capacity. We showed BWSTT to result in significant beneficial changes in treadmill walking ability, muscle fiber size and CS activity. From a

clinical perspective, this indicates that BWSTT in persons with a SCI is a form of rehabilitation that focuses on the recovery of both muscle strength and endurance. Finally, unlike forms of electrically stimulated training, BWSTT provides the individual with an incomplete SCI the opportunity to improve their gait during ambulation, therefore enabling much greater opportunity for improvements in independent walking capability.

Reference List

1. Aksnes, A. K., N. Hjeltnes, E. O. Wahlstrom, A. Katz, J. R. Zierath, and H. Wallberg-Henriksson. Intact glucose transport in morphologically altered denervated skeletal muscle from quadriplegic patients. *Am J Physiol* 271: E593-600, 1996.

2. Andersen, J. L., T. Mohr, F. Biering-Sorensen, H. Galbo, and M. Kjaer. Myosin heavy chain isoform transformation in single fibres from m. vastus lateralis in spinal cord injured individuals: effects of long- term functional electrical stimulation (FES). *Pflugers Arch* 431: 513-8, 1996.

3. Andersen, P. H., S. Lund, O. Schmitz, S. Junker, B. B. Kahn, and O. Pedersen. Increased insulin-stimulated glucose uptake in athletes: the importance of GLUT4 mRNA, GLUT4 protein and fibre type composition of skeletal muscle. *Acta Physiol Scand* 149: 393-404, 1993.

4. Anderson, J., M. I. Almeida-Silveira, and C. Perot. Reflex and muscular adaptations in rat soleus muscle after hindlimb suspension. *J Exp Biol* 202 ( Pt 19): 2701-7, 1999.

5. Baldwin, K. M. and F. Haddad. Effects of different activity and inactivity paradigms on myosin heavy chain gene expression in striated muscle. *J Appl Physiol* 90: 345-57, 2001.

6. Barbeau, H., M. Ladouceur, K. E. Norman, A. Pepin, and A. Leroux. Walking after spinal cord injury: evaluation, treatment, and functional recovery. *Arch Phys Med Rehabil* 80: 225-35, 1999.

7. Barbeau, H. and S. Rossignol. Enhancement of locomotor recovery following spinal cord injury. *Curr Opin Neurol* 7: 517-24, 1994.

8. Bauman, W. A., R. H. Adkins, A. M. Spungen, and R. L. Waters. The effect of residual neurological deficit on oral glucose tolerance in persons with chronic spinal cord injury. *Spinal Cord* 37: 765-71,

1999.

9. Bauman, W. A. and A. M. Spungen. Disorders of carbohydrate and lipid metabolism in veterans with paraplegia or quadriplegia: a model of premature aging. *Metabolism* 43: 749-56, 1994.

10. Blakemore, S. J., P. K. Rickhuss, P. W. Watt, M. J. Rennie, and H. S. Hundal. Effects of limb immobilization on cytochrome c oxidase activity and GLUT4 and GLUT5 protein expression in human skeletal muscle. *Clin Sci (Lond)* 91: 591-9, 1996.

11. Bonen, A., M. H. Tan, P. Clune, and R. L. Kirby. Effects of exercise on insulin binding to human muscle. *Am J Physiol* 248: E403-8, 1985.

12. Brooke, M. H. and K. K. Kaiser. Three "myosin adenosine triphosphatase" systems: the nature of their pH lability and sulfhydryl dependence. *J Histochem Cytochem* 18: 670-2, 1970.

13. Brooke, M. H. and K. K. Kaiser. Muscle fiber types: how many and what kind? *Arch Neurol* 23: 369-79, 1970.

14. Burnham, R., T. Martin, R. Stein, G. Bell, I. MacLean, and R. Steadward. Skeletal muscle fibre type transformation following spinal cord injury. *Spinal Cord* 35: 86-91, 1997.

15. Castro, M. J., D. F. Apple Jr, R. S. Staron, G. E. Campos, and G. A. Dudley. Influence of complete spinal cord injury on skeletal muscle within 6 mo of injury. *J Appl Physiol* 86: 350-8, 1999.

16. Chilibeck, P. D., G. Bell, J. Jeon, C. B. Weiss, G. Murdoch, I. MacLean, E. Ryan, and R. Burnham. Functional electrical stimulation exercise increases GLUT-1 and GLUT-4 in paralyzed skeletal muscle. *Metabolism* 48: 1409-13, 1999.

17. Chilibeck, P. D., J. Jeon, C. Weiss, G. Bell, and R. Burnham. Histochemical changes in muscle of individuals with spinal cord injury following functional electrical stimulated exercise training. *Spinal Cord* 37: 264-8, 1999.

18. Coggan, A. R., W. M. Kohrt, R. J. Spina, J. P. Kirwan, D. M. Bier, and J. O. Holloszy. Plasma glucose kinetics during exercise in subjects with high and low lactate thresholds. *J Appl Physiol* 73: 1873-80, 1992.

19. Cortez, M. Y., C. E. Torgan, J. T. Brozinick Jr, and J. L. Ivy. Insulin resistance of obese Zucker rats exercise trained at two different intensities. *Am J Physiol* 261: E613-9, 1991.

20. Daugaard, J. R., J. N. Nielsen, S. Kristiansen, J. L. Andersen, M. Hargreaves, and E. A. Richter. Fiber type-specific expression of GLUT4 in human skeletal muscle: influence of exercise training. *Diabetes* 49: 1092-5, 2000.

21. Daugaard, J. R. and E. A. Richter. Relationship between muscle fibre composition, glucose transporter protein 4 and exercise training: possible consequences in non-insulin- dependent diabetes mellitus. *Acta Physiol Scand* 171: 267-76, 2001.

22. Davies, H. Hope as a coping strategy for the spinal cord injured individual. *Axone* 15: 40-6, 1993.

23. Davis, G., M. J. Plyley, and R. J. Shephard. Gains of cardiorespiratory fitness with arm-crank training in spinally disabled men. *Can J Sport Sci* 16: 64-72, 1991.

24. Dela, F. On the influence of physical training on glucose homeostasis. *Acta Physiol Scand Suppl* 635: 1-41, 1996.

25. Dela, F., J. J. Larsen, K. J. Mikines, T. Ploug, L. N. Petersen, and H. Galbo. Insulin-stimulated

muscle glucose clearance in patients with NIDDM. Effects of one-legged physical training. *Diabetes* 44: 1010-20, 1995.

26. Dela, F., T. Ploug, A. Handberg, L. N. Petersen, J. J. Larsen, K. J. Mikines, and H. Galbo. Physical training increases muscle GLUT4 protein and mRNA in patients with NIDDM. *Diabetes* 43: 862-5, 1994.

27. Dietz, V., M. Wirz, and L. Jensen. Locomotion in patients with spinal cord injuries. *Phys Ther* 77: 508-16, 1997.

28. Duckworth, W. C., P. Jallepalli, and S. S. Solomon. Glucose intolerance in spinal cord injury. *Arch Phys Med Rehabil* 64: 107-10, 1983.

29. Duckworth, W. C., S. S. Solomon, P. Jallepalli, C. Heckemeyer, J. Finnern, and A. Powers. Glucose intolerance due to insulin resistance in patients with spinal cord injuries. *Diabetes* 29: 906-10, 1980.

30. Ferrando, A. A., K. D. Tipton, M. M. Bamman, and R. R. Wolfe. Resistance exercise maintains skeletal muscle protein synthesis during bed rest. *J Appl Physiol* 82: 807-10, 1997.

31. Field-Fote, E. C. Combined use of body weight support, functional electric stimulation, and treadmill training to improve walking ability in individuals with chronic incomplete spinal cord injury. *Arch Phys Med Rehabil* 82: 818-24, 2001.

32. Gao, J., J. Ren, E. A. Gulve, and J. O. Holloszy. Additive effect of contractions and insulin on GLUT-4 translocation into the sarcolemma. *J Appl Physiol* 77: 1597-601, 1994.

33. Gardner, M. B., M. K. Holden, J. M. Leikauskas, and R. L. Richard. Partial body weight support with treadmill locomotion to improve gait after incomplete spinal cord injury: a single-subject

experimental design. *Phys Ther* 78: 361-74, 1998.

34. Garland, D. E., C. A. Stewart, R. H. Adkins, S. S. Hu, C. Rosen, F. J. Liotta, and D. A. Weinstein. Osteoporosis after spinal cord injury. *J Orthop Res* 10: 371-8, 1992.

35. Gaster, M., P. Poulsen, A. Handberg, H. D. Schroder, and H. Beck-Nielsen. Direct evidence of fiber type-dependent GLUT-4 expression in human skeletal muscle. *Am J Physiol Endocrinol Metab* 278: E910-6, 2000.

36. Gerrits, H. L., M. T. Hopman, A. J. Sargeant, D. A. Jones, and A. De Haan. Effects of training on contractile properties of paralyzed quadriceps muscle. *Muscle Nerve* 25: 559-67, 2002.

37. Goodyear, L. J., M. F. Hirshman, E. D. Horton, S. M. Knutson, L. J. Wardzala, and E. S. Horton. Exercise training normalizes glucose metabolism in a rat model of impaired glucose tolerance. *Metabolism* 40: 455-64, 1991.

38. Goodyear, L. J., M. F. Hirshman, and E. S. Horton. Exercise-induced translocation of skeletal muscle glucose transporters. *Am J Physiol* 261: E795-9, 1991.

39. Goodyear, L. J. and B. B. Kahn. Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med* 49: 235-61, 1998.

40. Gordon, T. and J. Mao. Muscle atrophy and procedures for training after spinal cord injury. *Phys Ther* 74: 50-60, 1994.

41. Gregory, C. M., K. Vandenborne, and G. A. Dudley. Metabolic enzymes and phenotypic expression among human locomotor muscles. *Muscle Nerve* 24: 387-93, 2001.

42. Greve, J. M., R. Muszkat, B. Schmidt, J. Chiovatto, T. E. Barros Filho, and L. R. Batisttella. Functional electrical stimulation (FES): muscle histochemical analysis. *Paraplegia* 31: 764-70, 1993.

43. Hedman, A., P. E. Andersson, R. Reneland, and H. O. Lithell. Insulin-mediated changes in leg blood flow are coupled to capillary density in skeletal muscle in healthy 70-year-old men. *Metabolism* 50: 1078-82, 2001.

44. Henriksen, E. J., R. E. Bourey, K. J. Rodnick, L. Koranyi, M. A. Permutt, and J. O. Holloszy. Glucose transporter protein content and glucose transport capacity in rat skeletal muscles. *Am J Physiol* 259: E593-8, 1990.

45. Hespel, P., L. Vergauwen, K. Vandenberghe, and E. A. Richter. Important role of insulin and flow in stimulating glucose uptake in contracting skeletal muscle. *Diabetes* 44: 210-5, 1995.

46. Hjeltnes, N., D. Galuska, M. Bjornholm, A. K. Aksnes, A. Lannem, J. R. Zierath, and H. Wallberg-Henriksson. Exercise-induced overexpression of key regulatory proteins involved in glucose uptake and metabolism in tetraplegic persons: molecular mechanism for improved glucose homeostasis. *FASEB J* 12: 1701-12, 1998.

47. Houmard, J. A., P. C. Egan, P. D. Neufer, J. E. Friedman, W. S. Wheeler, R. G. Israel, and G. L. Dohm. Elevated skeletal muscle glucose transporter levels in exercise-trained middle-aged men. *Am J Physiol* 261: E437-43, 1991.

48. Houmard, J. A., M. S. Hickey, G. L. Tyndall, K. E. Gavigan, and G. L. Dohm. Seven days of exercise increase GLUT-4 protein content in human skeletal muscle. *J Appl Physiol* 79: 1936-8, 1995.

49. Houmard, J. A., T. Hortobagyi, P. D. Neufer, R. A. Johns, D. D. Fraser, R. G. Israel, and G. L. Dohm. Training cessation does not alter GLUT-4 protein levels in human skeletal muscle. *J Appl Physiol* 74: 776-81, 1993.

50. Houmard, J. A., G. L. Tyndall, J. B. Midyette, M. S. Hickey, P. L. Dolan, K. E. Gavigan, M. L. Weidner, and G. L. Dohm. Effect of reduced training and training cessation on insulin action and muscle GLUT-4. *J Appl Physiol* 81: 1162-8, 1996.

51. Hughes, V. A., M. A. Fiatarone, R. A. Fielding, B. B. Kahn, C. M. Ferrara, P. Shepherd, E. C. Fisher, R. R. Wolfe, D. Elahi, and W. J. Evans. Exercise increases muscle GLUT-4 levels and insulin action in subjects with impaired glucose tolerance. *Am J Physiol* 264: E855-62, 1993.

52. Ivy, J. L., J. T. Brozinick Jr, C. E. Torgan, and G. M. Kastello. Skeletal muscle glucose transport in obese Zucker rats after exercise training. *J Appl Physiol* 66: 2635-41, 1989.

53. James, D. E., K. M. Burleigh, L. H. Storlien, S. P. Bennett, and E. W. Kraegen. Heterogeneity of insulin action in muscle: influence of blood flow. *Am J Physiol* 251: E422-30, 1986.

54. Jeon, J. Y., C. B. Weiss, R. D. Steadward, E. Ryan, R. S. Burnham, G. Bell, P. Chilibeck, and G. D. Wheeler. Improved glucose tolerance and insulin sensitivity after electrical stimulation-assisted cycling in people with spinal cord injury. *Spinal Cord* 40: 110-7, 2002.

55. Johannsson, E., O. Waerhaug, and A. Bonen. Effect of cross-reinnervation on the expression of GLUT-4 and GLUT-1 in slow and fast rat muscles. *Am J Physiol* 270: R1355-60, 1996.

56. Kagaya, H., Y. Shimada, K. Sato, and M. Sato. Changes in muscle force following therapeutic electrical stimulation in patients with complete paraplegia. *Paraplegia* 34: 24-9, 1996.

57. Karlsson, A. K. Insulin resistance and sympathetic function in high spinal cord injury. *Spinal Cord* 37: 494-500, 1999.

58. Kern, M., J. A. Wells, J. M. Stephens, C. W. Elton, J. E. Friedman, E. B. Tapscott, P. H. Pekala, and G. L. Dohm. Insulin responsiveness in skeletal muscle is determined by glucose transporter (Glut4) protein level. *Biochem J* 270: 397-400, 1990.

59. Kjaer, M., F. Dela, F. B. Sorensen, N. H. Secher, J. Bangsbo, T. Mohr, and H. Galbo. Fatty acid kinetics and carbohydrate metabolism during electrical exercise in spinal cord-injured humans. *Am J Physiol Regul Integr Comp Physiol* 281: R1492-8, 2001.

60. Kjaer, M., T. Mohr, F. Biering-Sorensen, and J. Bangsbo. Muscle enzyme adaptation to training and tapering-off in spinal-cord- injured humans. *Eur J Appl Physiol* 84: 482-6, 2001.

61. Koval, J. A., R. A. DeFronzo, R. M. O'Doherty, R. Printz, H. Ardehali, D. K. Granner, and L. J. Mandarino. Regulation of hexokinase II activity and expression in human muscle by moderate exercise. *Am J Physiol* 274: E304-8, 1998.

62. Kruszynska, Y. T., M. I. Mulford, J. Baloga, J. G. Yu, and J. M. Olefsky. Regulation of skeletal muscle hexokinase II by insulin in nondiabetic and NIDDM subjects. *Diabetes* 47: 1107-13, 1998.

63. Lillioja, S., A. A. Young, C. L. Culter, J. L. Ivy, W. G. Abbott, J. K. Zawadzki, H. Yki-Jarvinen, L. Christin, T. W. Secomb, and C. Bogardus. Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. *J Clin Invest* 80: 415-24, 1987.

64. Lund, S., G. D. Holman, O. Schmitz, and O. Pedersen. Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proc Natl Acad Sci U S A* 92: 5817-21, 1995.

65. Mandarino, L. J., R. L. Printz, K. A. Cusi, P. Kinchington, R. M. O'Doherty, H. Osawa, C. Sewell, A. Consoli, D. K. Granner, and R. A. DeFronzo. Regulation of hexokinase II and glycogen synthase mRNA, protein, and activity in human muscle. *Am J Physiol* 269: E701-8, 1995.

66. Marette, A., J. M. Richardson, T. Ramlal, T. W. Balon, M. Vranic, J. E. Pessin, and A. Klip. Abundance, localization, and insulin-induced translocation of glucose transporters in red and white muscle. *Am J Physiol* 263: C443-52, 1992.

67. Martin, T. P., R. B. Stein, P. H. Hoeppner, and D. C. Reid. Influence of electrical stimulation on the morphological and metabolic properties of paralyzed muscle. *J Appl Physiol* 72: 1401-6, 1992.

68. McCoy, M., J. Proietto, and M. Hargreves. Effect of detraining on GLUT-4 protein in human skeletal muscle. *J Appl Physiol* 77: 1532-6, 1994.

69. Mikines, K. J. The influence of physical activity and inactivity on insulin action and secretion in man. *Acta Physiol Scand Suppl* 609: 1-43, 1992.

70. Mohr, T., J. L. Andersen, F. Biering-Sorensen, H. Galbo, J. Bangsbo, A. Wagner, and M. Kjaer. Long-term adaptation to electrically induced cycle training in severe spinal cord injured individuals. *Spinal Cord* 35: 1-16, 1997.

71. Mohr, T., F. Dela, A. Handberg, F. Biering-Sorensen, H. Galbo, and M. Kjaer. Insulin action and long-term electrically induced training in individuals with spinal cord injuries. *Med Sci Sports Exerc* 33: 1247-52, 2001.

72. Myllynen, P., V. A. Koivisto, and E. A. Nikkila. Glucose intolerance and insulin resistance accompany immobilization. *Acta Med Scand* 222: 75-81, 1987.

73. Pacy, P. J., R. Hesp, D. A. Halliday, D. Katz, G. Cameron, and J. Reeve. Muscle and bone in paraplegic patients, and the effect of functional electrical stimulation. *Clin Sci (Lond)* 75: 481-7, 1988.

74. Pette, D., H. Peuker, and R. S. Staron. The impact of biochemical methods for single muscle fibre analysis. *Acta Physiol Scand* 166: 261-77, 1999.

75. Pette, D. and R. S. Staron. Myosin isoforms, muscle fiber types, and transitions. *Microsc Res Tech* 50: 500-9, 2000.

76. Pette, D. and R. S. Staron. Transitions of muscle fiber phenotypic profiles. *Histochem Cell Biol* 115: 359-72, 2001.

77. Phillips, S. M., X. X. Han, H. J. Green, and A. Bonen. Increments in skeletal muscle GLUT-1 and GLUT-4 after endurance training in humans. *Am J Physiol* 270: E456-62, 1996.

78. Pinter, M. M. and M. R. Dimitrijevic. Gait after spinal cord injury and the central pattern generator for locomotion. *Spinal Cord* 37: 531-7, 1999.

79. Protas, E. J., S. A. Holmes, H. Qureshy, A. Johnson, D. Lee, and A. M. Sherwood. Supported treadmill ambulation training after spinal cord injury: a pilot study. *Arch Phys Med Rehabil* 82: 825-31, 2001.

80. Rasio, E. The capillary barrier to circulating insulin. *Diabetes Care* 5: 158-61, 1982.

81. Rea, S. and D. E. James. Moving GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles. *Diabetes* 46: 1667-77, 1997.

82. Richter, E. A., P. Jensen, B. Kiens, and S. Kristiansen. Sarcolemmal glucose transport and GLUT-4 translocation during exercise are diminished by endurance training. *Am J Physiol* 274: E89-95, 1998.

83. Ritov, V. B. and D. E. Kelley. Hexokinase isozyme distribution in human skeletal muscle. *Diabetes* 50: 1253-62, 2001.

84. Rochester, L., M. J. Barron, C. S. Chandler, R. A. Sutton, S. Miller, and M. A. Johnson. Influence of electrical stimulation of the tibialis anterior muscle in paraplegic subjects. 2. Morphological and histochemical properties. *Paraplegia* 33: 514-22, 1995.

85. Rose, A. J., K. Howlett, D. S. King, and M. Hargreaves. Effect of prior exercise on glucose metabolism in trained men. *Am J Physiol Endocrinol Metab* 281: E766-71, 2001.

86. Ross, I. B. and C. H. Tator. Spinal cord blood flow and evoked potential responses after treatment with nimodipine or methylprednisolone in spinal cord-injured rats. *Neurosurgery* 33: 470-6; discussion 476-7, 1993.

87. Round, J. M., F. M. Barr, B. Moffat, and D. A. Jones. Fibre areas and histochemical fibre types in the quadriceps muscle of paraplegic subjects. *J Neurol Sci* 116: 207-11, 1993.

88. Ryder, J. W., A. V. Chibalin, and J. R. Zierath. Intracellular mechanisms underlying increases in glucose uptake in response to insulin or exercise in skeletal muscle. *Acta Physiol Scand* 171: 249-57, 2001.

89. Ryder, J. W., M. Gilbert, and J. R. Zierath. Skeletal muscle and insulin sensitivity: pathophysiological alterations. *Front Biosci* 6: D154-63, 2001.

90. Saltzstein, R. J., S. Hardin, and J. Hastings. Osteoporosis in spinal cord injury: using an index of

mobility and its relationship to bone density. *J Am Paraplegia Soc* 15: 232-4, 1992.

91. Sato, Y. Diabetes and life-styles: role of physical exercise for primary prevention. *Br J Nutr* 84 Suppl 2: S187-90, 2000.

92. Scelsi, R., C. Marchetti, P. Poggi, S. Lotta, and G. Lommi. Muscle fiber type morphology and distribution in paraplegic patients with traumatic cord lesion. Histochemical and ultrastructural aspects of rectus femoris muscle. *Acta Neuropathol (Berl)* 57: 243-8, 1982.

93. Shepherd, P. R. and B. B. Kahn. Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. *N Engl J Med* 341: 248-57, 1999.

94. Shields, R. K. Muscular, skeletal, and neural adaptations following spinal cord injury. *J Orthop Sports Phys Ther* 32: 65-74, 2002.

95. Simoneau, J. A. and C. Bouchard. Genetic determinism of fiber type proportion in human skeletal muscle. *FASEB J* 9: 1091-5, 1995.

96. Sloan, K. E., L. A. Bremner, J. Byrne, R. E. Day, and E. R. Scull. Musculoskeletal effects of an electrical stimulation induced cycling programme in the spinal injured. *Paraplegia* 32: 407-15, 1994.

97. Staron, R. S., F. C. Hagerman, R. S. Hikida, T. F. Murray, D. P. Hostler, M. T. Crill, K. E. Ragg, and K. Toma. Fiber type composition of the vastus lateralis muscle of young men and women. *J Histochem Cytochem* 48: 623-9, 2000.

98. Tabata, I., Y. Suzuki, T. Fukunaga, T. Yokozeki, H. Akima, and K. Funato. Resistance training affects GLUT-4 content in skeletal muscle of humans after 19 days of head-down bed rest. *J Appl Physiol* 86: 909-14, 1999.

99. Talmadge, R. J. Myosin heavy chain isoform expression following reduced neuromuscular activity: potential regulatory mechanisms. *Muscle Nerve* 23: 661-79, 2000.

100. Talmadge, R. J., M. J. Castro, D. F. Apple Jr, and G. A. Dudley. Phenotypic adaptations in human muscle fibers 6 and 24 wk after spinal cord injury. *J Appl Physiol* 92: 147-54, 2002.

101. Tator, C. H., E. G. Duncan, V. E. Edmonds, L. I. Lapczak, and D. F. Andrews. Changes in epidemiology of acute spinal cord injury from 1947 to 1981. *Surg Neurol* 40: 207-15, 1993.

102. Tharion, G., K. R. Prasad, L. Gopalan, and S. Bhattacharji. Glucose intolerance and dyslipidaemias in persons with paraplegia and tetraplegia in south India. *Spinal Cord* 36: 228-30, 1998.

103. Treadway, J. L., D. E. James, E. Burcel, and N. B. Ruderman. Effect of exercise on insulin receptor binding and kinase activity in skeletal muscle. *Am J Physiol* 256: E138-44, 1989.

104. Vestergaard, H., C. Bjorbaek, T. Hansen, F. S. Larsen, D. K. Granner, and O. Pedersen. Impaired activity and gene expression of hexokinase II in muscle from non-insulin-dependent diabetes mellitus patients. *J Clin Invest* 96: 2639-45, 1995.

105. Vukovich, M. D., P. J. Arciero, W. M. Kohrt, S. B. Racette, P. A. Hansen, and J. O. Holloszy. Changes in insulin action and GLUT-4 with 6 days of inactivity in endurance runners. *J Appl Physiol* 80: 240-4, 1996.

106. Wallberg-Henriksson, H., S. H. Constable, D. A. Young, and J. O. Holloszy. Glucose transport into rat skeletal muscle: interaction between exercise and insulin. *J Appl Physiol* 65: 909-13, 1988.

107. Wernig, A. and S. Muller. Laufband locomotion with body weight support improved walking in persons with severe spinal cord injuries. *Paraplegia* 30: 229-38, 1992.

108. Wernig, A., S. Muller, A. Nanassy, and E. Cagol. Laufband therapy based on 'rules of spinal locomotion' is effective in spinal cord injured persons. *Eur J Neurosci* 7: 823-9, 1995.

109. Wernig, A., A. Nanassy, and S. Muller. Maintenance of locomotor abilities following Laufband (treadmill) therapy in para- and tetraplegic persons: follow-up studies. *Spinal Cord* 36: 744-9, 1998.

110. Williamson, D. L., P. M. Gallagher, C. C. Carroll, U. Raue, and S. W. Trappe. Reduction in hybrid single muscle fiber proportions with resistance training in humans. *J Appl Physiol* 91: 1955-61, 2001.

111. Wojtaszewski, J. F., B. F. Hansen, Gade, B. Kiens, J. F. Markuns, L. J. Goodyear, and E. A. Richter. Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. *Diabetes* 49: 325-31, 2000.

112. Wojtaszewski, J. F. and E. A. Richter. Glucose utilization during exercise: influence of endurance training. *Acta Physiol Scand* 162: 351-8, 1998.

113. Zierath, J. R., L. He, A. Guma, E. Odegoard Wahlstrom, A. Klip, and H. Wallberg-Henriksson. Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia* 39: 1180-9, 1996.

# APPENDIX 1

BLOOD GLUCOSE RAW DATA, PAIRED T-TEST, and ANOVA TABLE

1 = TREATMENT (TRAINING); 2 = TIME

# BLOOD GLUCOSE CONCENTRATION DURING OGTT

**PRE - TRAINING**

| Time | 0 | 10 | 20 | 40 | 60 | 80 | 100 | 120 |
|---|---|---|---|---|---|---|---|---|
| S1 | 3.99 | 4.7 | 5.53 | 5.77 | 5.79 | 4.52 | 5.44 | 4.64 |
| S2 | 3.77 | 3.9 | 6.22 | 7.74 | 8.65 | 8.45 | 10 | 10.8 |
| S3 | 4.22 | 4.81 | 6.12 | 7.26 | 6.26 | 5.12 | 5.27 | 3.79 |
| S4 | 4.76 | 5.56 | 6.36 | 6.93 | 6.35 | 6.09 | 5.65 | 8.02 |
| S5 | 3.67 | 6.49 | 7.46 | 7.67 | 6.64 | 6.47 | 4.65 | 4.93 |
| S6 | 3.8 | 5.16 | 5.45 | 4.56 | 5.25 | 5.09 | 6.03 | 5.51 |
| S7 | 4.56 | 5.08 | 6.62 | 7.43 | 7.21 | 6.18 | 5.65 | 5.23 |
| S8 | 4.32 | 4.97 | 6 | 6.42 | 6.34 | 4.38 | 5.27 | 5.82 |
| S9 | 4.51 | 4.84 | 5.29 | 6.78 | 8.08 | 8.49 | 8.57 | 8.38 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mean | 4.13625 | 5.08375 | 6.22 | 6.7225 | 6.56125 | 5.7875 | 5.995 | 6.0925 |
| SD | 0.39558 | 0.741291 | 0.637473 | 1.098099 | 1.020398 | 1.326647 | 1.666802 | 2.261351 |
| SE | 0.13186 | 0.247097 | 0.212491 | 0.366033 | 0.340133 | 0.442216 | 0.555601 | 0.753784 |

| Time | 140 | 160 | 180 |
|---|---|---|---|
| S1 | 4.5 | 4.77 | 4.4 |
| S2 | 10.3 | 10.1 | 8.94 |
| S3 | 4.78 | 3.62 | 2.58 |
| S4 | 7.06 | 6.26 | 5.35 |
| S5 | 5.36 | 5.04 | |
| S6 | 5.88 | 5.18 | 4.51 |
| S7 | 6.05 | | 5.01 |
| S8 | 6.14 | 6.21 | 5.25 |
| S9 | 8.52 | 7.62 | 5.51 |

| | | | |
|---|---|---|---|
| Mean | 6.25875 | 5.882857 | 5.148571 |
| SD | 1.822968 | 2.065468 | 1.915319 |
| SE | 0.607656 | 0.730253 | 0.677168 |

# BLOOD GLUCOSE CONCENTRATION DURING OGTT

**POST - TRAINING**

| Time | 0 | 10 | 20 | 40 | 60 | 80 | 100 | 120 |
|---|---|---|---|---|---|---|---|---|
| S1 | 3.98 | 4.47 | 4.93 | 5.79 | 5.68 | 4.54 | 5.09 | 3.79 |
| S2 | 3.89 | 4.91 | 6.88 | 7.44 | 7.37 | 7.46 | 8.07 | 8.92 |
| S3 | 3.35 | 4.21 | 5.23 | 6.9 | 5.65 | 5.14 | 3.96 | 3.49 |
| S4 | 4.47 | 5.27 | 6.06 | 6.42 | 6.27 | 6.68 | 7.71 | 8.17 |
| S5 | 5.03 | 5.36 | 5.89 | 7.66 | 6.46 | 5.87 | 5.58 | 5.71 |
| S6 | 3.38 | 4.58 | 5.36 | 5.31 | 5.04 | 5.6 | 4.96 | 5.2 |
| S7 | 4.35 | 4.53 | 4.84 | 6.2 | 6.68 | 6.47 | 6.38 | 6.74 |
| S8 | 4.52 | 5.13 | 6.06 | 6.1 | 5.97 | 6.27 | 6.33 | 6.98 |
| S9 | 4.46 | 5.04 | 5.49 | 6.12 | 6.03 | 5.69 | 5.25 | 5.3 |
| Mean | 4.158889 | 4.833333 | 5.637778 | 6.437778 | 6.127778 | 5.968889 | 5.925556 | 6.033333 |
| SD | 0.582665 | 0.419753 | 0.690899 | 0.807266 | 0.71857 | 0.920853 | 1.399449 | 1.946667 |
| SE | 0.194222 | 0.139918 | 0.2303 | 0.269089 | 0.239523 | 0.306951 | 0.466483 | 0.648889 |

| Time | 140 | 160 | 180 |
|---|---|---|---|
| S1 | 4.34 | 3.97 | 4.47 |
| S2 | 9.24 | 8.3 | 7.71 |
| S3 | 3.47 | 3.19 | 3.7 |
| S4 | 6.13 | 5.59 | 5.69 |
| S5 | 5.4 | 5.52 | |
| S6 | 5.04 | 4.82 | 5.21 |
| S7 | 6.69 | 6.39 | 5.19 |
| S8 | 1.7 | | 2.98 |
| S9 | 5.15 | 5.01 | 5.1 |
| Mean | 5.24 | 5.34875 | 5.00625 |
| SD | 2.249879 | 1.668629 | 1.524868 |
| SE | 0.74996 | 0.58995 | 0.539122 |

**Glucose ANOVA for all time points**

Summary of all Effects; design: (blood glucose 2.sta)
1-TRAINING, 2-TIME

| | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | 1 | 7.36586 | 6 | 0.833058 | 8.84195 | 0.024843 |
| 2 | 10 | 7.913821 | 60 | 1.656011 | 4.778844 | 4.94E-05 |
| 12 | 10 | 0.482295 | 60 | 0.223561 | 2.157336 | 0.033191 |

# OGTT BLOOD GLUCOSE SUMMARY

**Blood glucose AUC values**

| | Pre | Post | %Change |
|---|---|---|---|
| **S1** | 902.5 | 847.3 | -6.11634 |
| **S2** | 1561 | 1385 | -11.2748 |
| **S3** | 908.8 | 810.3 | -10.8385 |
| **S4** | 1156 | 1162 | 0.519031 |
| **S5** | 960 | 955.9 | -0.42708 |
| **S6** | 947.5 | 914.6 | -3.4723 |
| **S7** | 1334 | 1103 | -17.3163 |
| **S8** | 1089 | 908.4 | -16.584 |
| **S9** | 1025 | 967.1 | -5.64878 |
| **MEAN** | 1098.2 | 1005.956 | -7.90657 |
| **STDEV** | 222.0782 | 181.2364 | 6.506554 |
| **SE** | 74.02606 | 60.41214 | 2.168851 |

t-Test: Paired Two Sample for Means

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 1098.2 | 1005.956 |
| Variance | 49318.72 | 32846.64 |
| Observations | 9 | 9 |
| Pearson Correlation | 0.931264 | |
| Hypothesized Mean Difference | 0 | |
| Df | 8 | |
| t Stat | 3.26108 | |
| P(T<=t) one-tail | 0.005754 | |
| t Critical one-tail | 1.859548 | |
| P(T<=t) two-tail | 0.011509 | |
| t Critical two-tail | 2.306006 | |

# APPENDIX 2

PLASMA INSULIN RAW DATA, INSULIN RESISTANCE INDEX,
PAIRED T-TEST, and ANOVA TABLE

1 = TREATMENT (TRAINING); 2 = TIME

# INSULIN CONCENTRATION DURING OGTT

**PRE - TRAINING**

| Time | S1 | S2 | S3 | S4 | S5 | S6 |
|---|---|---|---|---|---|---|
| 0 | 1.040799 | 4.087584 | 2.656848 | 2.125038 | 0.9466 | 2.257182 |
| 20 | 12.17774 | 70.62633 | 5.04158 | 31.6422 | 9.492195 | 51.1407 |
| 40 | 14.75933 | 121.4973 | 15.04412 | 37.32215 | 13.44209 | 14.91649 |
| 60 | 21.93142 | 122.5933 | 34.43235 | 17.29285 | 21.74536 | 41.27306 |
| 120 | 19.61402 | 235.3784 | 27.52749 | 75.8694 | 15.9172 | 55.42002 |
| 180 | 10.94628 | 255.6034 | 10.65042 | 9.800916 | 12.51603 | 20.79058 |
| **AUC** | **2932** | **30580** | **3777** | **6938** | **2668** | **6944** |

| Time | S7 | S8 | S9 |
|---|---|---|---|
| 0 | 2.833783 | 1.221987 | 10.31731 |
| 20 | 27.1373 | 8.105255 | 53.9842 |
| 40 | 48.21328 | 11.44898 | 54.3584 |
| 60 | 69.56114 | 7.127983 | 107.1684 |
| 120 | 91.99552 | 24.80086 | 212.2104 |
| 180 | 36.08001 | 11.82678 | 130.0369 |
| **AUC** | **10920** | **2531** | **23190** |

| MEAN | SD | SE |
|---|---|---|
| 3.054126 | 2.902123 | 0.967374 |
| 29.9275 | 23.7803 | 7.926766 |
| 36.77802 | 35.77486 | 11.92495 |
| 49.2362 | 41.4244 | 13.80813 |
| 84.3037 | 83.4969 | 27.8323 |
| 55.36126 | 84.42359 | 28.1412 |
| **10053.33** | **10095.02** | **3365.005** |

**POST - TRAINING**

| Time | S1 | S2 | S3 | S4 | S5 | S6 |
|---|---|---|---|---|---|---|
| 0 | 1.097657 | 4.845813 | 1.18759 | 3.500795 | 1.279872 | 2.766092 |
| 20 | 3.376499 | 79.02539 | 2.20073 | 26.78963 | 4.694255 | 31.72975 |
| 40 | 7.574721 | 118.3768 | 25.16325 | 33.17162 | 10.65532 | 25.26776 |
| 60 | 11.09856 | 112.763 | 27.10608 | 40.71615 | 9.610958 | 16.98896 |
| 120 | 11.22966 | 195.8243 | 29.18547 | 108.8345 | 11.21158 | 37.34794 |
| 180 | 8.633098 | 197.7728 | 23.98097 | 24.76091 | 5.896125 | 30.3296 |
| **AUC** | **1607** | **26190** | **4114** | **10140** | **1554** | **4998** |

| Time | S7 | S8 | S9 |
|---|---|---|---|
| 0 | 2.46358 | 2.060946 | 8.129553 |
| 20 | 17.37267 | 3.236433 | 29.91346 |
| 40 | 22.81743 | 6.205355 | 83.72722 |
| 60 | 12.41557 | 8.913878 | 96.97654 |
| 120 | 90.7124 | 10.04307 | 106.5287 |
| 180 | 5.773866 | 3.080113 | 123.5853 |
| **AUC** | **6941** | **1261** | **16330** |

| MEAN | SD | SE |
|---|---|---|
| 3.036878 | 2.265082 | 0.755027 |
| 22.03765 | 24.60674 | 8.202246 |
| 36.9955 | 38.41773 | 12.80591 |
| 37.39885 | 39.7841 | 13.26137 |
| 66.76863 | 63.48829 | 21.16276 |
| 47.09031 | 67.72198 | 22.57399 |
| **8126.111** | **8349.936** | **2783.312** |

Summary of all Effects; design:
1-TRAINING, 2-TIME

| df Effect | MS Effect | Df Error | MS Error | F | p-level |
|---|---|---|---|---|---|
| 1 | 1 | 1541.307 | 8 319.3269 | 4.826736 | 0.059272 |
| 2 | 5 | 10688.35 | 40 1804.142 | 5.924337 | 0.000345 |
| 12 | 5 | 212.2145 | 40 213.9375 | 0.991946 | 0.434878 |

# OGTT PLASMA INSULIN SUMMARY

**Insulin AUC values**

| | Pre | Post | %Change |
|---|---|---|---|
| **S1** | 2932 | 1607 | -45.191 |
| **S2** | 30580 | 26190 | -14.3558 |
| **S3** | 3777 | 4114 | 8.922425 |
| **S4** | 6938 | 10140 | 46.15163 |
| **S5** | 2668 | 1554 | -41.7541 |
| **S6** | 6944 | 4998 | -28.0242 |
| **S7** | 10920 | 6941 | -36.4377 |
| **S8** | 2531 | 1261 | -50.1778 |
| **S9** | 23190 | 16330 | -29.5817 |
| **MEAN** | 10053.33 | 8126.111 | -20.1083 |
| **STDEV** | 9419.822 | 8298.491 | 32.94459 |
| **SE** | 3139.941 | 2766.164 | 10.98153 |

t-Test: Paired Two Sample for Means

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 10053.33 | 8126.111 |
| Variance | 1.02E+08 | 69721429 |
| Observations | 9 | 9 |
| Pearson Correlation | 0.968016 | |
| Hypothesized Mean Difference | 0 | |
| df | 8 | |
| t Stat | 1.990437 | |
| P(T<=t) one-tail | 0.040859 | |
| t Critical one-tail | 1.859548 | |
| P(T<=t) two-tail | 0.081718 | |
| t Critical two-tail | 2.306006 | |

# INSULIN RESISTANCE INDEX

| | Pre | Post | %Change |
|---|---|---|---|
| **S1** | 2646130 | 1361611 | -48.5433 |
| **S2** | 47735380 | 36273150 | -24.012 |
| **S3** | 3432538 | 3333574 | -2.8831 |
| **S4** | 8020328 | 11782680 | 46.9102 |
| **S5** | 2561280 | 1485469 | -42.0029 |
| **S6** | 6579440 | 4571171 | -30.5234 |
| **S7** | 14567280 | 7655923 | -47.4444 |
| **S8** | 2756259 | 1145492 | -58.4403 |
| **S9** | 23769750 | 15792743 | -33.5595 |
| **MEAN** | 12452043 | 9266868 | -26.7221 |
| **STDEV** | 15384812 | 11827292 | 34.12391 |
| **SE** | 5128271 | 3942431 | 11.37464 |

t-Test: Paired Two Sample for Means

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 12452043 | 9266868 |
| Variance | 2.25E+14 | 1.28E+14 |
| Observations | 9 | 9 |
| Pearson Correlation | 0.975317 | |
| Hypothesized Mean Difference | 0 | |
| df | 8 | |
| t Stat | 2.042623 | |
| P(T<=t) one-tail | 0.037683 | |
| t Critical one-tail | 1.859548 | |
| P(T<=t) two-tail | 0.075366 | |
| t Critical two-tail | 2.306006 | |

# APPENDIX 3

HISTOCHEMISTRY RAW DATA and PAIRED T-TEST

## Computer Analysis of mATPase Stain

**Example: Subject # S1 at 200x magnification and pH 4.6**

| Picture 1 | Dark fibers | | Light fibers | | Intermediate fibers | |
|---|---|---|---|---|---|---|
| | **Stats** | **Area** | **Stats** | **Area** | **Stats** | **Area** |
| | Min | 2012.471 | Min | 758.4888 | Min | 977.8378 |
| | (Obj.#) | 11 | (Obj.#) | 14 | (Obj.#) | 20 |
| | Max | 5484.845 | Max | 7765.347 | Max | 6410.923 |
| | (Obj.#) | 8 | (Obj.#) | 5 | (Obj.#) | 11 |
| | Range | 3472.374 | Range | 7006.858 | Range | 5433.085 |
| | Mean | 3441.9 | Mean | 4433.107 | Mean | 3251.032 |
| | Std.Dev | 1116.204 | Std.Dev | 1692.176 | Std.Dev | 1568.101 |
| | Sum | 41302.81 | Sum | 66496.61 | Sum | 81275.8 |
| | Samples | 12 | Samples | 15 | Samples | 25 |
| **Picture 2** | **Dark fibers** | | **Light fibers** | | **Intermediate fibers** | |
| | **Stats** | **Area** | **Stats** | **Area** | **Stats** | **Area** |
| | Min | 2407.803 | Min | 1746.119 | Min | 2175.304 |
| | (Obj.#) | 13 | (Obj.#) | 14 | (Obj.#) | 6 |
| | Max | 5010.335 | Max | 5807.433 | Max | 6439.181 |
| | (Obj.#) | 11 | (Obj.#) | 10 | (Obj.#) | 7 |
| | Range | 2602.532 | Range | 4061.314 | Range | 4263.876 |
| | Mean | 3390.187 | Mean | 4507.392 | Mean | 3712.8 |
| | Std.Dev | 630.6322 | Std.Dev | 971.8261 | Std.Dev | 1257.277 |
| | Sum | 67803.74 | Sum | 72118.27 | Sum | 44553.59 |
| | Samples | 20 | Samples | 16 | Samples | 12 |
| **Picture 3** | **Dark fibers** | | **Light fibers** | | **Intermediate fibers** | |
| | **Stats** | **Area** | **Stats** | **Area** | **Stats** | **Area** |
| | Min | 2452.009 | Min | 2377.027 | Min | 1589.441 |
| | (Obj.#) | 5 | (Obj.#) | 12 | (Obj.#) | 1 |
| | Max | 6201.926 | Max | 7056.379 | Max | 5666.703 |
| | (Obj.#) | 12 | (Obj.#) | 14 | (Obj.#) | 4 |
| | Range | 3749.917 | Range | 4679.353 | Range | 4077.262 |
| | Mean | 4036.973 | Mean | 4362.839 | Mean | 3720.764 |
| | Std.Dev | 896.8601 | Std.Dev | 1425.753 | Std.Dev | 1285.238 |
| | Sum | 76702.48 | Sum | 61079.75 | Sum | 37207.64 |
| | Samples | 19 | Samples | 14 | Samples | 10 |
| **Picture4** | **Dark fibers** | | **Light fibers** | | **Intermediate fibers** | |
| | **Stats** | **Area** | **Stats** | **Area** | **Stats** | **Area** |
| | Min | 1013.93 | Min | 2688.984 | Min | 2008.554 |
| | (Obj.#) | 20 | (Obj.#) | 7 | (Obj.#) | 8 |
| | Max | 3505.388 | Max | 4926.68 | Max | 4676.555 |
| | (Obj.#) | 11 | (Obj.#) | 14 | (Obj.#) | 2 |
| | Range | 2491.458 | Range | 2237.696 | Range | 2668.001 |
| | Mean | 2460.57 | Mean | 4153.313 | Mean | 3677.677 |
| | Std.Dev | 720.6751 | Std.Dev | 612.1569 | Std.Dev | 907.2194 |
| | Sum | 49211.4 | Sum | 70606.32 | Sum | 36776.77 |
| | Samples | 20 | Samples | 17 | Samples | 10 |

# FIBER-TYPE DATA ANALYSIS

## 1) MEAN FIBER SIZE

| | Type 1 | | |
|---|---|---|---|
| | Pre | Post | %Change |
| S1 | 3310.1 | 3668.012 | 10.81274 |
| S2 | 2283.981 | 3832.597 | 67.8034 |
| S4 | 5261.449 | 4636.937 | -11.8696 |
| S5 | 2081.118 | 5638.873 | 170.954 |
| S6 | 2925.288 | 5773.35 | 97.36009 |
| S7 | 5613.5 | 7480.325 | 33.25599 |
| S8 | 2467.532 | 4486.755 | 81.83173 |
| Mean | 3420.4 | 5073.8 | 64.3 |
| SD | 1440.459 | 1332.437 | 61.12023 |
| SE | 544.4424 | 503.614 | 23.10128 |

| | Type 2a | | |
|---|---|---|---|
| | Pre | Post | %Change |
| S1 | 4503.974 | 4340.514 | -3.62925 |
| S2 | 3235.204 | 4959.846 | 53.30859 |
| S4 | 6438.792 | 6700.687 | 4.067456 |
| S5 | 1895.662 | 6739.693 | 255.5324 |
| S6 | 2819.862 | 3214.505 | 13.99512 |
| S7 | 7828.5 | 7478.471 | -4.47121 |
| S8 | 2951.89 | 6057.381 | 105.2035 |
| Mean | 4239.1 | 5641.6 | 60.6 |
| SD | 2158.829 | 1523.31 | 94.66507 |
| SE | 815.9605 | 575.7572 | 35.78003 |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 3420.424 | 5073.836 |
| Variance | 2074922 | 1775389 |
| Observations | 7 | 7 |
| Pearson Correlation | 0.477189 | |
| Hypothesized Mean Difference | 0 | |
| Df | 6 | |
| t Stat | -3.079 | |
| P(T<=t) one-tail | 0.010845 | |
| t Critical one-tail | 1.943181 | |
| P(T<=t) two-tail | 0.021691 | |
| t Critical two-tail | 2.446914 | |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 4239.126 | 5641.585 |
| Variance | 4660541 | 2320474 |
| Observations | 7 | 7 |
| Pearson Correlation | 0.486475 | |
| Hypothesized Mean Difference | 0 | |
| df | 6 | |
| t Stat | -1.90815 | |
| P(T<=t) one-tail | 0.052488 | |
| t Critical one-tail | 1.943181 | |
| P(T<=t) two-tail | 0.104977 | |
| t Critical two-tail | 2.446914 | |

**1) MEAN FIBER SIZE**

| | Type 2x | | |
|---|---|---|---|
| | **Pre** | **Post** | **%Change** |
| **S1** | 3419.979 | 3657.828 | 6.954694 |
| **S2** | 2507.585 | 2813.064 | 12.18222 |
| **S4** | 3920.101 | 4008.073 | 2.244126 |
| **S5** | 2536.736 | 6593.378 | 159.9158 |
| **S6** | 2889.188 | 4747.539 | 64.32089 |
| **S7** | 6244.8 | 5657.75 | -9.40063 |
| **S8** | 2943.575 | 4252.617 | 44.47117 |
| **Mean** | 3494.6 | 4532.9 | 40.1 |
| **SD** | 1310.984 | 1267.183 | 58.76929 |
| **SE** | 495.5054 | 478.9501 | 22.21271 |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 3494.566 | 4532.893 |
| Variance | 1718679 | 1605752 |
| Observations | 7 | 7 |
| Pearson Correlation | 0.269263 | |
| Hypothesized Mean Difference | 0 | |
| Df | 6 | |
| t Stat | -1.76237 | |
| P(T<=t) one-tail | 0.064236 | |
| t Critical one-tail | 1.943181 | |
| P(T<=t) two-tail | 0.128473 | |
| t Critical two-tail | 2.446914 | |

**2) % DISTRIBUTION**

| | Type 1 | | |
|---|---|---|---|
| | **Pre** | **Post** | **Change** |
| **S1** | 37.17277 | 28.05755 | -9.11522 |
| **S2** | 30.87558 | 26.95652 | -3.91905 |
| **S4** | 26.79739 | 42.64706 | 15.84967 |
| **S5** | 11.90476 | 13.70968 | 1.804916 |
| **S6** | 5.494505 | 6.077348 | 0.582843 |
| **S7** | 9.448819 | 13.75 | 4.301181 |
| **S8** | 9.177215 | 22.97297 | 13.79576 |
| **Mean** | 18.69586 | 22.02445 | 3.328585 |
| **SD** | 12.59553 | 12.10253 | 8.986309 |
| **SE** | 4.760662 | 4.574326 | 3.396506 |

| | Type 2a | | |
|---|---|---|---|
| | **Pre** | **Post** | **Change** |
| **S1** | 13.08901 | 30.93525 | 17.84625 |
| **S2** | 23.96313 | 20 | -3.96313 |
| **S4** | 30.06536 | 27.20588 | -2.85948 |
| **S5** | 19.04762 | 59.67742 | 40.6298 |
| **S6** | 46.15385 | 66.29834 | 20.1445 |
| **S7** | 15.74803 | 38.75 | 23.00197 |
| **S8** | 43.67089 | 51.35135 | 7.680465 |
| **Mean** | 27.39113 | 42.03118 | 14.64005 |
| **SD** | 13.20164 | 17.4461 | 15.74122 |
| **SE** | 4.98975 | 6.594006 | 5.94962 |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 18.69586 | 22.02445 |
| Variance | 158.6473 | 146.4712 |
| Observations | 7 | 7 |
| Pearson Correlation | 0.735923 | |
| Hypothesized Mean Difference | 0 | |
| Df | 6 | |
| t Stat | -0.98 | |
| P(T<=t) one-tail | 0.182466 | |
| t Critical one-tail | 1.943181 | |
| P(T<=t) two-tail | 0.364932 | |
| t Critical two-tail | 2.446914 | |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 27.39113 | 42.03118 |
| Variance | 174.2832 | 304.3664 |
| Observations | 7 | 7 |
| Pearson Correlation | 0.501187 | |
| Hypothesized Mean Difference | 0 | |
| df | 6 | |
| t Stat | -2.46067 | |
| P(T<=t) one-tail | 0.024537 | |
| t Critical one-tail | 1.943181 | |
| P(T<=t) two-tail | 0.049075 | |
| t Critical two-tail | 2.446914 | |

**2) % DISTRIBUTION**

| | Type 2ax | | |
|---|---|---|---|
| | Pre | Post | Change |
| S1 | 28.27225 | 24.82014 | -3.45211 |
| S2 | 9.21659 | 14.78261 | 5.566019 |
| S4 | 7.189542 | 2.941176 | -4.24837 |
| S5 | 60.47619 | 14.51613 | -45.9601 |
| S6 | 20.14652 | 16.0221 | -4.12442 |
| S7 | 0 | 35 | 35 |
| S8 | 22.1519 | 3.378378 | -18.7735 |
| Mean | 21.06471 | 15.92293 | -5.14178 |
| SD | 19.93263 | 11.34516 | 24.43585 |
| SE | 7.533826 | 4.288068 | 9.235883 |

| | Type 2x | | |
|---|---|---|---|
| | Pre | Post | Change |
| S1 | 21.46597 | 16.18705 | -5.27892 |
| S2 | 35.9447 | 38.26087 | 2.316169 |
| S4 | 35.94771 | 27.20588 | -8.74183 |
| S5 | 8.571429 | 12.09677 | 3.525346 |
| S6 | 28.20513 | 11.60221 | -16.6029 |
| S7 | 74.80315 | 12.5 | -62.3031 |
| S8 | 25 | 22.2973 | -2.7027 |
| Mean | 32.8483 | 20.02144 | -12.8269 |
| SD | 20.7424 | 9.953483 | 22.85835 |
| SE | 7.839889 | 3.762063 | 8.639645 |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 21.06471 | 15.92293 |
| Variance | 397.3098 | 128.7127 |
| Observations | 7 | 7 |
| Pearson Correlation | -0.15718 | |
| Hypothesized Mean Difference | 0 | |
| Df | 6 | |
| t Stat | 0.556718 | |
| P(T<=t) one-tail | 0.298928 | |
| t Critical one-tail | 1.943181 | |
| P(T<=t) two-tail | 0.597856 | |
| t Critical two-tail | 2.446914 | |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 32.8483 | 20.02144 |
| Variance | 430.247 | 99.07182 |
| Observations | 7 | 7 |
| Pearson Correlation | 0.016504 | |
| Hypothesized Mean Difference | 0 | |
| df | 6 | |
| t Stat | 1.484651 | |
| P(T<=t) one-tail | 0.094086 | |
| t Critical one-tail | 1.943181 | |
| P(T<=t) two-tail | 0.188173 | |
| t Critical two-tail | 2.446914 | |

**3) % AREA**

| | Type 1 | | |
|---|---|---|---|
| | **Pre** | **Post** | **Change** |
| **S1** | 33.17742 | 26.88337 | -6.29405 |
| **S2** | 27.20722 | 28.08952 | 0.882303 |
| **S4** | 27.56162 | 39.03613 | 11.47451 |
| **S5** | 11.49003 | 11.87582 | 0.38579 |
| **S6** | 5.56318 | 9.815476 | 4.252296 |
| **S7** | 8.243123 | 13.54185 | 5.29873 |
| **S8** | 8.232012 | 19.85929 | 11.62728 |
| **Mean** | 17.35352 | 21.30021 | 3.946694 |
| **SD** | 11.48398 | 10.60516 | 6.383889 |
| **SE** | 4.340536 | 4.008372 | 2.412883 |

| | Type 2a | | |
|---|---|---|---|
| | **Pre** | **Post** | **Change** |
| **S1** | 31.46177 | 46.93273 | 15.47096 |
| **S2** | 33.96408 | 34.80878 | 0.8447 |
| **S4** | 41.36681 | 37.71219 | -3.65463 |
| **S5** | 47.58588 | 68.82918 | 21.24329 |
| **S6** | 55.63907 | 67.19729 | 11.55821 |
| **S7** | 19.15974 | 57.65041 | 38.49067 |
| **S8** | 55.93946 | 60.90115 | 4.961685 |
| **Mean** | 40.73098 | 53.4331 | 12.70213 |
| **SD** | 13.52757 | 13.76176 | 14.23044 |
| **SE** | 5.11294 | 5.201457 | 5.378599 |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 17.35352 | 21.30021 |
| Variance | 131.8818 | 112.4693 |
| Observations | 7 | 7 |
| Pearson Correlation | 0.835857 | |
| Hypothesized Mean Difference | 0 | |
| Df | 6 | |
| t Stat | -1.63568 | |
| P(T<=t) one-tail | 0.076512 | |
| t Critical one-tail | 1.943181 | |
| P(T<=t) two-tail | 0.153024 | |
| t Critical two-tail | 2.446914 | |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 40.73098 | 53.4331 |
| Variance | 182.9951 | 189.3861 |
| Observations | 7 | 7 |
| Pearson Correlation | 0.456255 | |
| Hypothesized Mean Difference | 0 | |
| df | 6 | |
| t Stat | -2.36161 | |
| P(T<=t) one-tail | 0.02808 | |
| t Critical one-tail | 1.943181 | |
| P(T<=t) two-tail | 0.056161 | |
| t Critical two-tail | 2.446914 | |

**3) % AREA**

| | Type 2x | | |
|---|---|---|---|
| | **Pre** | **Post** | **Change** |
| **S1** | 35.36081 | 26.1839 | -9.17691 |
| **S2** | 38.8287 | 37.1017 | -1.727 |
| **S4** | 31.07157 | 23.25168 | -7.81988 |
| **S5** | 40.92409 | 19.295 | -21.6291 |
| **S6** | 38.79775 | 22.98724 | -15.8105 |
| **S7** | 72.59713 | 28.80773 | -43.7894 |
| **S8** | 35.82853 | 19.23956 | -16.589 |
| **Mean** | 41.91551 | 25.26669 | -16.6488 |
| **SD** | 13.89964 | 6.254213 | 13.65382 |
| **SE** | 5.253571 | 2.36387 | 5.160658 |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 41.91551 | 25.26669 |
| Variance | 193.2001 | 39.11518 |
| Observations | 7 | 7 |
| Pearson Correlation | 0.263935 | |
| Hypothesized Mean Difference | 0 | |
| Df | 6 | |
| t Stat | 3.226104 | |
| P(T<=t) one-tail | 0.008999 | |
| t Critical one-tail | 1.943181 | |
| P(T<=t) two-tail | 0.017998 | |
| t Critical two-tail | 2.446914 | |

# APPENDIX 4

GLUT 4 PROTEIN CONTENT

RAW DATA and PAIRED T-TEST

# GLUT 4 PROTEIN CONTENT

**Total Optical Density**

| | Pre | Post | %Change |
|---|---|---|---|
| **S1** | 109.801 | 175.449 | 59.78816 |
| **S2** | 61.136 | 177.135 | 189.7393 |
| **S3** | 60.0039 | 227.806 | 279.652 |
| **S4** | 136.396 | 228.668 | 67.65008 |
| **S5** | 177.402 | 162.638 | -8.32234 |
| **S6** | 92.4886 | 152.473 | 64.85599 |
| **S7** | 70.513 | 296.776 | 320.8813 |
| **S8** | 322.257 | 451.793 | 40.19649 |
| **Mean** | 128.7497 | 234.0923 | 126.8051 |
| **Stdev** | 88.02079 | 99.88735 | 121.0532 |
| **SE** | 31.12005 | 35.31551 | 42.79878 |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 128.7497 | 234.0923 |
| Variance | 7747.66 | 9977.483 |
| Observations | 8 | 8 |
| Pearson Correlation | 0.703749 | |
| Hypothesized Mean Difference | 0 | |
| Df | 7 | |
| t Stat | -4.07347 | |
| P(T<=t) one-tail | **0.002364** | |
| t Critical one-tail | 1.894578 | |
| P(T<=t) two-tail | 0.004728 | |
| t Critical two-tail | 2.364623 | |

# APPENDIX 5

CITRATE SYNTHASE PROTEIN CONTENT

RAW DATA and PAIRED T-TEST

# CITRATE SYNTHASE PROTEIN CONTENT

**Total Optical Density**

| | Pre | Post | %Change |
|---|---|---|---|
| **S1** | 170.031 | 171.366 | 0.785151 |
| **S2** | 146.961 | 258.642 | 75.99363 |
| **S3** | 79.2994 | 250.406 | 215.7729 |
| **S4** | 168.938 | 469.356 | 177.8274 |
| **S5** | 43.1344 | 72.9473 | 69.1163 |
| **S6** | 34.572 | 164.471 | 375.7347 |
| **S7** | 50.1509 | 230.808 | 360.227 |
| **S8** | 161.476 | 291.742 | 80.67205 |
| **Mean** | 106.8203 | 238.7173 | 169.5161 |
| **Stdev** | 60.58908 | 115.9621 | 139.5378 |
| **SE** | 21.42147 | 40.99878 | 49.33405 |

t-Test: Paired Two Sample for Means

| | Variable 1 | Variable 2 |
|---|---|---|
| Mean | 106.8203 | 238.7173 |
| Variance | 3671.036 | 13447.2 |
| Observations | 8 | 8 |
| Pearson Correlation | 0.606219 | |
| Hypothesized Mean Difference | 0 | |
| Df | 7 | |
| t Stat | -4.02291 | |
| P(T<=t) one-tail | **0.00252** | |
| t Critical one-tail | 1.894578 | |
| P(T<=t) two-tail | 0.005041 | |
| t Critical two-tail | 2.364623 | |

# APPENDIX 6

CITRATE SYNTHASE ACTIVITY ASSAY

RAW DATA AND PAIRED T-TEST

# CITRATE SYNTHASE ACTIVITY DATA

| | Pre | Post | %Change |
|---|---|---|---|
| **S1** | 7.883201 | 8.954678 | 13.5919 |
| **S2** | 6.035249 | 7.887765 | 30.69494 |
| **S3** | 5.283598 | 8.128613 | 53.84615 |
| **S4** | 5.584601 | 4.997702 | -10.5092 |
| **S5** | 8.6676 | 10.58799 | 22.15601 |
| **S6** | 10.4658 | 11.71875 | 11.97183 |
| **S7** | 5.733367 | 9.84375 | 71.69231 |
| **S8** | 5.544355 | 5.362426 | -3.28133 |
| **Mean** | 6.899722 | 8.43521 | 23.77032 |
| **Stdev** | 1.893435 | 2.370115 | 27.77798 |
| **SE** | 0.66943 | 0.837962 | 9.820999 |

t-Test: Paired Two Sample for Means

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 6.899722 | 8.43521 |
| Variance | 3.585095 | 5.617443 |
| Observations | 8 | 8 |
| Pearson Correlation | 0.765194 | |
| Hypothesized Mean Difference | 0 | |
| Df | 7 | |
| t Stat | -2.84235 | |
| P(T<=t) one-tail | **0.01248** | |
| t Critical one-tail | 1.894578 | |
| P(T<=t) two-tail | 0.02496 | |
| t Critical two-tail | 2.364623 | |

# APPENDIX 7

CAPILLARIZATION

RAW DATA AND PAIRED T-TEST

# CAPILLARIZATION ANALYSIS

### Capillaries per fiber

| | Pre | Post | %Change |
|---|---|---|---|
| S2 | 0.567 | 0.552 | -2.64091 |
| S4 | 0.826 | 0.7625 | -7.65278 |
| S5 | 0.642 | 0.6375 | -0.6686 |
| S6 | 0.816 | 0.75 | -8.03571 |
| S7 | 0.678 | 0.662338 | -2.26481 |
| S8 | 0.878 | 0.963964 | 9.758273 |
| Mean | 0.734 | 0.721 | -1.91742 |
| SD | 0.122739 | 0.141745 | 6.463023 |
| SE | 0.050108 | 0.057867 | 2.638518 |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 0.734378 | 0.721438 |
| Variance | 0.015065 | 0.020092 |
| Observations | 6 | 6 |
| Pearson Correlation | 0.923552 | |
| Hypothesized Mean Difference | 0 | |
| Df | 5 | |
| t Stat | 0.576659 | |
| P(T<=t) one-tail | 0.294578 | |
| t Critical one-tail | 2.015049 | |
| P(T<=t) two-tail | 0.589157 | |
| t Critical two-tail | 2.570578 | |

### Capillary density

| | Pre | Post | %Change |
|---|---|---|---|
| S2 | 112.168 | 109.851 | -2.0661 |
| S4 | 112.9955 | 76.99753 | -31.8579 |
| S5 | 261.0895 | 64.22508 | -75.4011 |
| S6 | 245.9852 | 167.0393 | -32.0937 |
| S7 | 101.9463 | 65.21055 | -36.0344 |
| S8 | 295.7324 | 204.7847 | -30.7534 |
| Mean | 188.319 | 114.685 | -34.7011 |
| SD | 88.42102 | 58.81593 | 23.46819 |
| SE | 36.09773 | 24.0115 | 9.580849 |

t-Test: Paired

| | *Variable 1* | *Variable 2* |
|---|---|---|
| Mean | 188.3195 | 114.6846 |
| Variance | 7818.277 | 3459.314 |
| Observations | 6 | 6 |
| Pearson Correlation | 0.634744 | |
| Hypothesized Mean Difference | 0 | |
| Df | 5 | |
| t Stat | 2.637807 | |
| P(T<=t) one-tail | 0.023049 | |
| t Critical one-tail | 2.015049 | |
| P(T<=t) two-tail | 0.046098 | |
| t Critical two-tail | 2.570578 | |

# APPENDIX 8

AMBULATION RAW DATA AND ANOVA TABLE

1 = TIME

# SUBJECT BACKGROUND AND PERFORMANCE DATA

**Walking Speed**

| | Speed | | |
|---|---|---|---|
| Subject | 0 Months | 6 Months | % Change |
| S1 | 0.8 | 1.5 | 87.5 |
| S2 | 1 | 1.5 | 50 |
| S3 | 1 | 1.7 | 70 |
| S4 | 0.5 | 1.1 | 120 |
| S5 | 0.1 | 0.7 | 600 |
| S6 | 0.4 | 0.8 | 100 |
| S7 | 0.5 | 1.1 | 120 |
| S8 | 0.3 | 1.2 | 300 |
| S9 | 0.3 | 1.1 | 266.6667 |
| **Average** | **0.54** | **1.19** | **190.46** |
| STDEV | 0.32059 | 0.329562 | 176.2392 |
| SE | 0.106863 | 0.109854 | 58.74641 |

**Duration**

| | Duration | | |
|---|---|---|---|
| Subject | 0 Months | 6 Months | % Change |
| S1 | 45 | 60 | 33.33333 |
| S2 | 30 | 35 | 16.66667 |
| S3 | 30 | 30 | 0 |
| S4 | 13 | 30 | 130.7692 |
| S5 | 10 | 26 | 160 |
| S6 | 30 | 30 | 0 |
| S7 | 15 | 32 | 113.3333 |
| S8 | 15 | 39 | 160 |
| S9 | 17 | 36 | 111.7647 |
| **Average** | **22.78** | **35.33** | **80.65** |
| STDEV | 11.5301537 | 10.0374299 | 67.53026 |
| SE | 3.84338457 | 3.34580998 | 22.51009 |

**% Body-weight Support**

| | % BWS | | |
|---|---|---|---|
| Subject | 0 months | 6 months | %Change |
| S1 | 49 | 0 | -100 |
| S2 | 63 | 31 | -50.7937 |
| S3 | 93 | 13 | -86.0215 |
| S4 | 42 | 0 | -100 |
| S5 | 65 | 40 | -38.4615 |
| S6 | 62 | 30 | -51.6129 |
| S7 | 75 | 24 | -68 |
| S8 | 76 | 41 | -46.0526 |
| S9 | 60 | 33 | -45 |
| **Average** | **65** | **23.56** | **-65.10** |
| STDEV | 15.11622 | 15.74096 | 24.3545 |
| SE | 5.038739 | 5.246986 | 8.118166 |

**Modified Wernig Scale results**

| Subject | Pre-test | 6 month | |
|---|---|---|---|
| S1 | | 4 | 4 |
| S2 | | 7 | 7 |
| S3 | | 0 | 4 |
| S4 | | 8 | 7 |
| S5 | | 0 | 7 |
| S6 | | 0 | 0 |
| S7 | | 0 | 2 |
| S8 | | 0 | 0 |
| S9 | | 0 | 0 |

**3 subjects (S3, S5, S7) improved functional classification of locomotor ability**

## Treadmill Performance Summary

**Speed (km/h)**

| | |
|---|---|
| Pre- test | 0.544 |
| 3 Months | 0.989 |
| 6 Months | 1.189 |

Summary of all Effects; design: (new.sta)
1-TIME

| | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | 2 | 0.979259 | 16 | 0.02175926 | 45.0042572 | 2.693E-07 |

Tukey HSD test; variable Var.1 (new.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: TIME

| Month | | 0 | 3 | 6 |
|---|---|---|---|---|
| | | {1} .5444444 | {2} .9888889 | {3} 1.188889 |
| 1 | {1} | | 0.000184 | 0.000168 |
| 2 | {2} | 0.000184 | | 0.028101 |
| 3 | {3} | 0.000168 | 0.028101 | |

**Duration (minutes)**

| | |
|---|---|
| Pre-test | 22.778 |
| 3 Months | 30.444 |
| 6 Months | 35.333 |

Summary of all Effects; design: (new.sta)
1-TIME

| | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | 2 | 360.4815 | 16 | 34.18982 | 10.54353 | 0.0012 |

Tukey HSD test; variable Var.1 (new.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: TIME

| Month | | 0 | 3 | 6 |
|---|---|---|---|---|
| | | {1} 22.77778 | {2} 30.44444 | {3} 35.33333 |
| 1 | {1} | | 0.03389 | 0.001014 |
| 2 | {2} | 0.03389 | | 0.209964 |
| 3 | {3} | 0.001014 | 0.209964 | |

## Treadmill Performance Summary

**% Body-weight Support**

| | |
|---|---|
| Pre -test | 65 |
| 3 months | 41.111 |
| 6 months | 23.556 |

Summary of all Effects; design: (new.sta)
1-TIME

| | df Effect | MS Effect | df Error | MS Error | F | p-level |
|---|---|---|---|---|---|---|
| 1 | 2 | 3894.778 | 16 | 102.944443 | 37.8337822 | 8.6146E-07 |

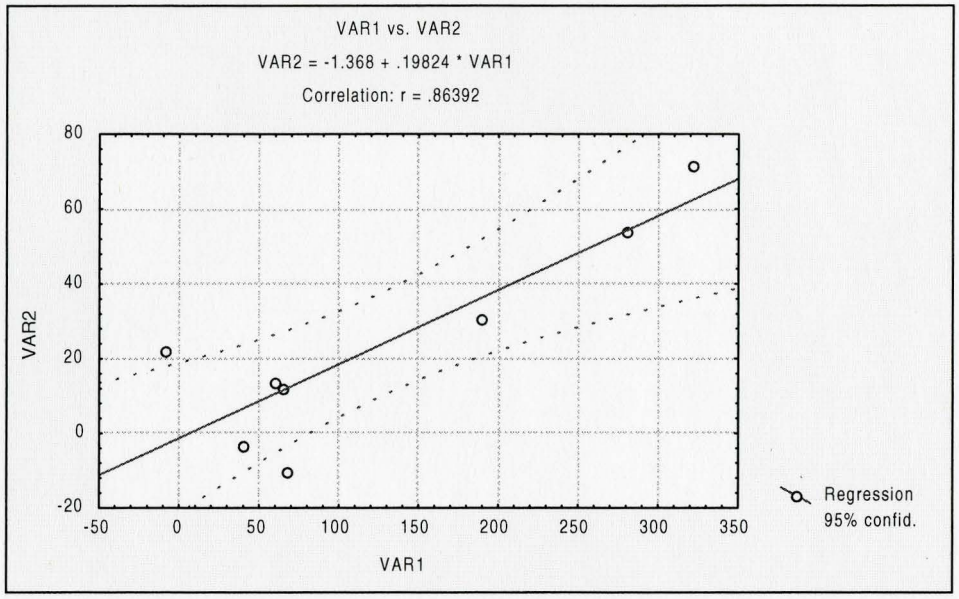
Tukey HSD test; variable Var.1 (new.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: TIME

| Month | | 0 | 3 | 6 |
|---|---|---|---|---|
| | | {1} | {2} | {3} |
| | | 65.00000 | 41.11111 | 23.55556 |
| 1 | {1} | | 0.0005 | 0.000168 |
| 2 | {2} | 0.0005 | | 0.005657 |
| 3 | {3} | 0.000168 | 0.005657 | |

# APPENDIX 9

CORRELATIONS and INDIVIDUAL SUBJECT DATA SUMMARY

## Correlation: GLUT 4 and CS ACTIVITY




| 1) | GLUT 4 | and | CS Activity |
|---|---|---|---|
| **S1** | 59.78816 | | 13.5919 |
| **S2** | 189.7393 | | 30.69494 |
| **S3** | 279.652 | | 53.84615 |
| **S4** | 67.65008 | | -10.5092 |
| **S5** | -8.32234 | | 22.15601 |
| **S6** | 64.85599 | | 11.97183 |
| **S7** | 320.8813 | | 71.69231 |
| **S8** | 40.19649 | | -3.28133 |

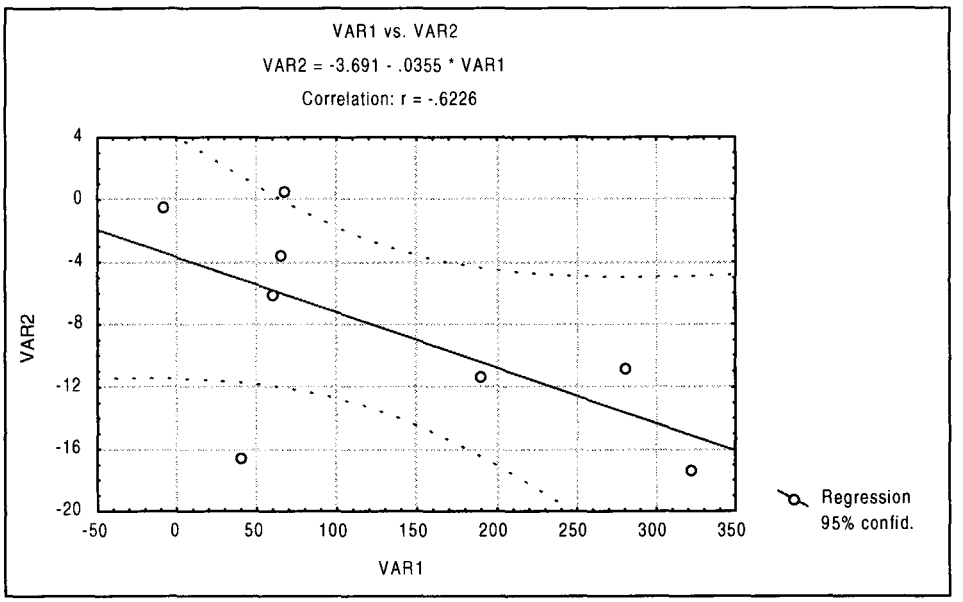
SUMMARY OUTPUT

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.863919 |
| R Square | 0.746357 |
| Adjusted R Square | 0.704083 |
| Standard Error | 65.85087 |
| Observations | 8 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 76559.17 | 76559.17 | 17.65526 | 0.005674 |
| Residual | 6 | 26018.02 | 4336.337 | | |
| Total | 7 | 102577.2 | | | |

## Correlation: GLUT 4 and AUC BLOOD GLUCOSE




| 2) | GLUT 4 | and | AUC Blood glucose |
|---|---|---|---|
| **S1** | 59.78816 | | -6.11634 |
| **S2** | 189.7393 | | -11.2748 |
| **S3** | 279.652 | | -10.8385 |
| **S4** | 67.65008 | | 0.519031 |
| **S5** | -8.32234 | | -0.42708 |
| **S6** | 64.85599 | | -3.4723 |
| **S7** | 320.8813 | | -17.3163 |
| **S8** | 40.19649 | | -16.584 |

SUMMARY OUTPUT

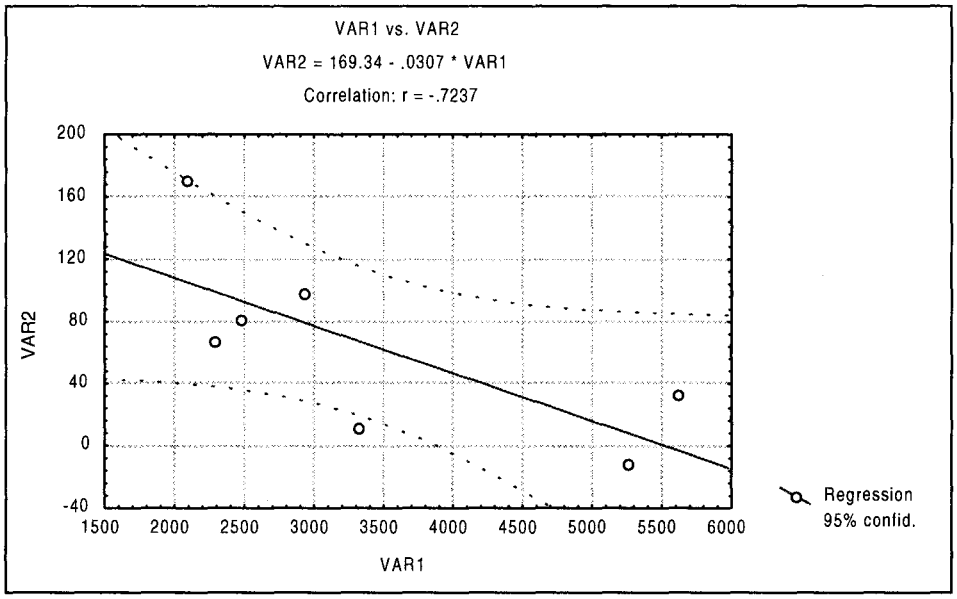
| *Regression Statistics* | |
|---|---|
| Multiple R | 0.62261 |
| R Square | 0.387643 |
| Adjusted R Square | 0.285583 |
| Standard Error | 102.318 |
| Observations | 8 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 39763.33 | 39763.33 | 3.798206 | 0.09921 |
| Residual | 6 | 62813.86 | 10468.98 | | |
| Total | 7 | 102577.2 | | | |

## Correlation: PRE-TRAINING FIBER SIZE AND PERCENT CHANGE

### Type 1

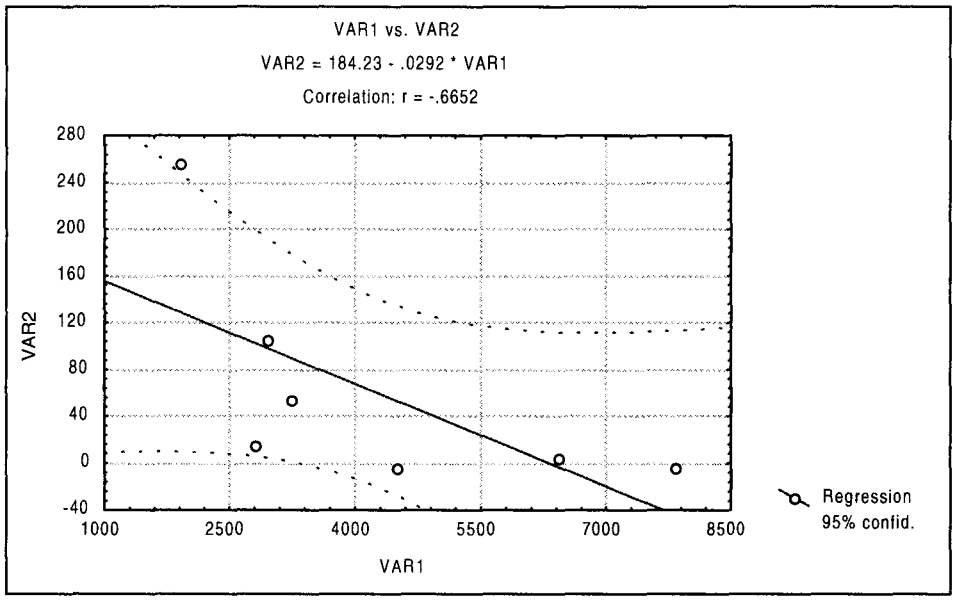



| *Regression Statistics* | |
|---|---|
| Multiple R | -0.723733 |
| R Square | 0.523789 |
| Adjusted R Square | 0.428547 |
| Standard Error | 1088.908 |
| Observations | 7 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 6520933 | 6520933 | 5.499554 | 0.06596 |
| Residual | 5 | 5928601 | 1185720 | | |
| Total | 6 | 12449534 | | | |

## Type IIa

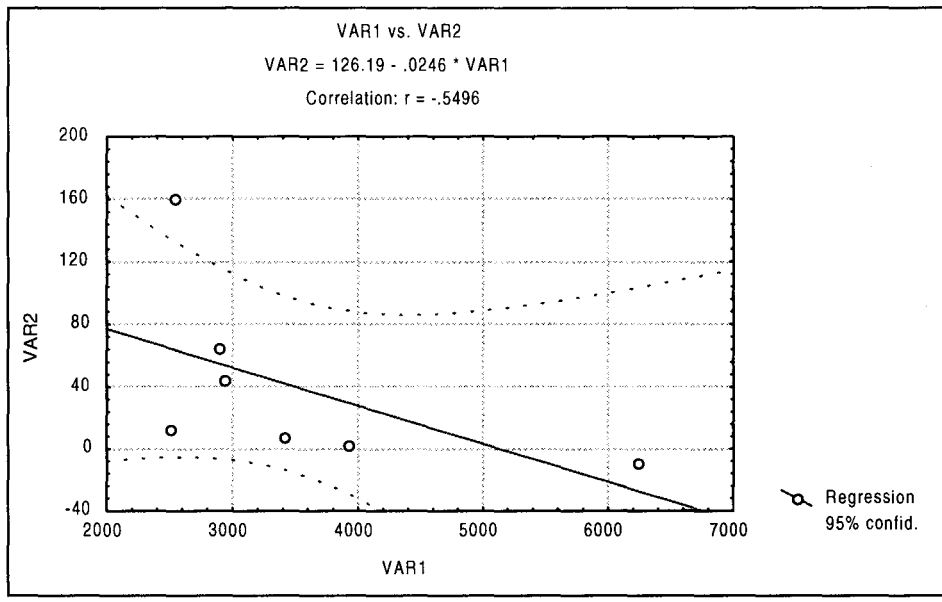



| *Regression Statistics* | |
|---|---|
| Multiple R | -0.665226 |
| R Square | 0.442526 |
| Adjusted R Square | 0.331031 |
| Standard Error | 1765.718 |
| Observations | 7 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 12374452 | 12374452 | 3.969022 | 0.102955 |
| Residual | 5 | 15588795 | 3117759 | | |
| Total | 6 | 27963247 | | | |

## Type IIx




| *Regression Statistics* | |
|---|---|
| Multiple R | -0.549578 |
| R Square | 0.302035 |
| Adjusted R Square | 0.162443 |
| Standard Error | 1199.788 |
| Observations | 7 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 3114612 | 3114612 | 2.163688 | 0.20127 |
| Residual | 5 | 7197462 | 1439492 | | |
| Total | 6 | 10312074 | | | |

# INDIVIDUAL SUBJECT DATA

**Change in:**

| Subject | Glucose AUC | Insulin AUC | Type I Fiber Size | Type IIa Fiber Size | Type IIa Proportion | Type IIx Proportion | GLUT 4 |
|---|---|---|---|---|---|---|---|
| S1 | -6.11634 | -45.190996 | 10.8127386 | -3.629248 | 15.470962 | -9.1769106 | 59.78816 |
| S2 | -11.2748 | -14.355788 | 67.8033953 | 53.308585 | 0.8446996 | -1.7270021 | 189.7393 |
| S3 | -10.8385 | 8.9224252 | | | | | 279.652 |
| S4 | 0.519031 | 46.151629 | -11.869593 | 4.0674556 | -3.6546266 | -7.8198845 | 67.65008 |
| S5 | -0.42708 | -41.754123 | 170.953959 | 255.53236 | 21.243294 | -21.629085 | -8.32234 |
| S6 | -3.4723 | -28.024194 | 97.3600892 | 13.995125 | 11.558215 | -15.810511 | 64.85599 |
| S7 | -17.3163 | -36.437729 | 33.2559946 | -4.471211 | 38.490669 | -43.789399 | 320.8813 |
| S8 | -16.584 | -50.177795 | 81.831735 | 105.20351 | 4.961685 | -16.588965 | 40.19649 |
| S9 | -5.64878 | -29.581716 | | | | | |

| Subject | CS | CS activity | C/F | Treadmill Speed | Walking Duration | BWS |
|---|---|---|---|---|---|---|
| S1 | 0.785151 | 13.591902 | | 87.5 | 33.333333 | -100 |
| S2 | 75.99363 | 30.694942 | -2.6409145 | 50 | 16.666667 | -50.793651 |
| S3 | 215.7729 | 53.846154 | | 70 | 0 | -86.021505 |
| S4 | 177.8274 | -10.50923 | -7.6527778 | 120 | 130.76923 | -100 |
| S5 | 69.1163 | 22.156007 | -0.6686047 | 600 | 160 | -38.461538 |
| S6 | 375.7347 | 11.971831 | -8.0357143 | 100 | 0 | -51.612903 |
| S7 | 360.227 | 71.692308 | -2.2648084 | 120 | 113.33333 | -68 |
| S8 | 80.67205 | -3.2813341 | 9.75827312 | 300 | 160 | -46.052632 |
| S9 | | | | 266.66667 | 111.76471 | -45 |

# APPENDIX 10

HISTOCHEMISTRY PROTOCOL

## Muscle Preparation

### Part A: Cutting Muscle

1. Store OCT mounted muscle at -70°C.
2. Prior to cutting, place muscle in cryostat for at least 15 min and let it warm up (soften). If muscle is not allowed to thaw, it will crack and/or chip, causing you to lose muscle. Set cryostat temperature to -20°C.
3. Cut sections to 8μm thick for H&E stain and to 10μm for mATPase and α-amylase PAS stain.
4. Cut two muscle tissue samples per slide. Allow to dry for an hour minimum. Place in -4°C to -20°C fridge while drying. Alternatively, allow to dry for 20 min. minimum at room temperature, and wrap tightly in aluminum foil and place in freezer for storage. Cut slides will stay good in freezer for approximately 3 wks. When removing from freezer to begin staining, let dry for minimum 25 minutes.
5. Perform HEMATOXYLIN AND EOSIN (H+E) STAINING:

   H+E stain is used to check for correct fiber orientation (fibers should be cross-sectional, not "parallel" when mounted and cut onto slides). "Parallel" fibers appear long and thin when stained. H+E also check for freezing artifact. If freezing artifact is present, cut deeper into the muscle and redo the H+E stain. If less than 50% of the fibers are affected by freezing artifact after the 2nd H+E stain, the muscle is suitable for further cutting and staining.

   a) fix cut sections in absolute ethanol for at least 3 minutes.
   b) Place slides in luke warm water for 30 sec.
   c) Rinse in $ddH_2O$ (one dunk is fine).
   d) Stain with Mayer's Hematoxylin solution for 1 min. (Sigma; MHS-16)
   e) Place in tap water for 30 sec.
   f) Place in 0.05M TBS (Tris buffered saline) solution (pH 7.6) for 1 min.
   g) Place in water for 30 sec.
   h) Dunk in $ddH_2O$.
   i) Stain with Eosin for between 30sec-1min. (will have to play with the timing – I'd say start with 35 seconds, seemed to work better). Eosin needs to be acidified with 0.5ml of glacial acetic acid per 100ml (Sigma; HT110-2-16)
   j) Place in water for 30 sec.
   k) Dehydrate slides: -place in 70% ethanol for 20 sec.
      -place in 95% ethanol for 20 sec.
      -place in 100% ethanol for 20 sec.
   l) place in xylene kept in fume hood for 30 seconds.
   m) Place cover slips on slides using permount. One small drop of permount is sufficient. Let permount dry for 30 min. before evaluating under microscope. Find picture at 100x magnification. Evaluate at 200x magnification.

# MUSCLE FIBRE TYPE ANALYSIS

Adapted from R.J. Snow - School of Health Sciences, Deakin University, Australia

PART A: PREPARATION OF SOLUTIONS

## Alkaline Stock Solution, pH 9.4

| | Reagent | Manufacturer | Amount for 250 mL | Amount for 500 mL | Amount for 1000mL |
|---|---|---|---|---|---|
| 1 | Glycine | BioShop Biotechnology Grade – GLN 001 | 1.40815 g | 2.8163 g | 5.6326 g |
| 2 | $CaCl_2 2H_2O$ | BDH 10070 | 1.50 g | 3.00 g | 6.00 g |
| 3 | NaCl | BioShop Reagent Grade – SOD 002 | 1.0969 g | 2.1938 g | 4.3876 g |
| 4 | NaOH | BDH Analytical Reagent | 0.6750 g | 1.3500 g | 2.70 g |
| 5 | MilliQ $H_2O$ | | 250 mL | 500 mL | 1000mL |

1. Dissolve reagents in MilliQ $H_20$ and bring to volume.
2. Calibrate pH meter prior to adjusting pH to 9.4 with HCl/5M KOH.
3. Store stock solution in fridge.

## Acid Preincubation Stock Solution, pH 4.6

| | Reagent | Manufacturer | Amount for 250 mL | Amount for 500 mL | Amount for 1000mL |
|---|---|---|---|---|---|
| 1 | Potassium Acetate | EM PX 1330-1 | 1.225 g | 2.45 g | 4.90 g |
| 2 | $CaCl_2 2H_2O$ | BDH 10070 | 0.65 g | 1.30 g | 2.60 g |
| 3 | MilliQ $H_2O$ | | 250 mL | 500 mL | 1000 mL |

1. Dissolve reagents in MilliQ $H_2O$ and bring to volume.
2. Calibrate pH meter prior to adjusting pH to 4.6 with glacial acetic acid.
3. Store stock solution in fridge.

5M NaOH (MW: 40.00 g/mol) – Dissolve 20.00 g in 100 mL

5M KOH (MW: 56.11 g/mol) – Dissolve 28.055 g in 100 mL

**Alkaline Preincubation Solution**

1. Remove alkaline stock solution from fridge and allow stock solution to get to room temperature.
2. Adjust pH of an appropriate volume (50mL) of alkaline stock solution to 10.50? using 5M NaOH (This should be done with continuous mixing and a pH meter sensitive to 0.001 pH units).

**Acid Preincubation Solution**

1. Remove acid stock solution from fridge and allow stock solution to get to room temperature.
2. Adjust pH of an appropriate volume (50mL) of acid stock solution to 4.30, 4.54, 4.60 with glacial acetic acid (This should be done with continuous mixing and a pH meter sensitive to 0.001 pH units).

**ATP Preincubation Solution, pH 9.4 (PREPARE FRESH DAILY)**

1. Add 170 mg of ATP (SIGMA A2383) to 100mL volumetric flask and bring up to volume using ALKALINE STOCK SOLUTION.
2. Adjust pH to 9.4.
3. Keep in fridge until ready for use.

**1% Calcium Chloride Stock Solution**

1. Dissolve 10 g of $CaCl_2H_2O$ in 1000mL volumetric flask using MilliQ $H_2O$ and bring up to volume.
2. Store at room temperature.

**2% Cobalt Chloride**

1. Dissolve 5 g of $CoCl_2.6H_2O$ in 250mL volumetric flask using MilliQ $H_2O$ and bring up to volume.
2. Cover in aluminum foil and store at room temperature.

**1% Ammonium Sulfide (PREPARE FRESH DAILY)**

1. Add 5 mL of 20% ammonium sulfide solution to 100mL volumetric flask.
2. Bring to volume.
3. Store in fume hood until ready for use.

PART B: FIBRE TYPE PROCEDURE

1. Cut tissue to a thickness of 10μm with cryostat.
2. Place 2-3 slices per slide.
3. Allow tissue sections to adhere to the slide at 4°C overnight.

4. Incubate the sections in acid preincubation solutions adjusted to a pH of 4.30, 4.54 and 4.60; and alkaline preincubation solution adjusted to pH of 10.50?? at the following time periods:

| pH | Incubation time (min) |
|---|---|
| 10.50 | 25 |
| 4.54 | 7.5 |
| 4.60 | 6.5 |
| 4.30 | 5.0 |

5. Transfer slides into plastic staining trough.

6. Rinse slides in distilled water **3 times.**

7. **Incubate** slides in **ATP incubation solution** for **45 minutes at 37°C**. This should be done in a temperature-controlled shaker.

8. **Rinse** slides in distilled water **2 times**.

9. **Incubate** slides in **1% $CaCl_2.2H_20$** (Calcium Chloride) for **3 minutes** at room temperature.

10. **Rinse** slides in distilled water **5 times.**

11. **Incubate** slides in **2% $CoCl_2 \cdot 6H_20$** (Cobalt Chloride) for 3 minutes at room temperature.

12. **Rinse** slides with distilled water **5 times.**

13. **Incubate** slides in **1% ammonium sulphide** for **1 minute** at room temperature.

14. **Rinse** slides in distilled water **5 times.**

15. **Dehydrate tissue** for **2 minutes** in each alcohol concentrations (70, 80, 90, 95 and 100% ethanol).

16. **Clear** sections with **xylene**. Do this **twice** in clean xylene @ **2 minutes.**

17. **Mount** the coverslips on slides using Permount (Fisher SP15-100). Store slides in the dark when not in use.

# APPENDIX 11

WESTERN BLOT PROTOCOL

# GENERAL WESTERN BLOT PROTOCOL USED IN SCI PROTEIN ANALYSIS (2002)

Adapted from Doug Mahoney (2001)

***For skeletal muscle biopsies:***

**(1) Homogenize muscle**
**(2) Determine protein concentration (Bradford assay)**
**(3) SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**
**(4) Western blotting**
**(5) Densitometry**

## HOMOGENIZATION

**Do all work in the fume hood.
*Ingredients:*
Homogenization Buffer:

1.211 g (10mM) tris(hydroxymethy)aminomethane (Tris base)
1.902 g (5mM) ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)
15.4 mg (0.1 mM) dithiothreitol (DTT)
0.348 g (2mM) phenylmethylsulfonyl fluoride (PMSF)
5 µl of a 25 mg/ml solution (0.125 µg/ml) leupeptin
0.5 g (0.5%) sodium lauryl sulfate (SDS)
Mix ingredients, fill to 900 mL with $dH_20$, and pH to 7.5. Fill to a final volume of 1 L.

*Instructions:*
(1) Weigh muscle sample and record.
(2) Add corresponding amount of homogenizing buffer. See below:

| Muscle Weights | Homogenization Buffer |
|---|---|
| 20 mg | 350 µl |
| 30 mg | 425 µl |
| 40 mg | 500 µl |
| 50 mg | 600 µl |
| 60 mg | 700 µl |
| 70 mg | 800 µl |
| 80 mg | 900 µl |
| 90 mg | 1000 µl |
| 100 mg | 1100 µl |

(3) Homogenize 100 passes with the chilled pestle in a chilled glass homogenizer and transfer to an eppendorf.
(4) Centrifuge at 15 000 rpm for 15 minutes at 4°C.
(5) Transfer the supernatant to an eppendorf and store in the –70°C until ready for analysis.

## PROTEIN CONTENT DETERMINATION

The Bio-Rad protein assay (#500-0006, Bio-Rad, Hercules, CA) (aka Bradford Protein Assay) is used for protein content determination. It is very important that this be done accurately, as it is imperative that equal amounts of protein are loaded in each lane for electrophoresis (see next step)

1. A standard curve using bovine serum albumin (BSA) (Boehringer-Mannheim; 735-078) is run with each new microtitre plate. Each point on the calibration curve is run in triplicate.
   a) Make up a 10mg/ml BSA protein standard (100mg BSA in 10 ml $ddH_2O$). Dilute further to yield desired protein concentrations.
   b) Calibration curve protein concentrations:

| *Desired Protein Concentration (mg/ml)* | *Volume of 10mg/ml BSA Standard (ml)* | *Volume of $ddH_20$ (ml)* |
|---|---|---|
| 10.0 | 1.00 | 0 |
| 7.5 | 0.75 | 0.25 |
| 5.0 | 0.50 | 0.50 |
| 2.5 | 0.25 | 0.75 |
| 1.0 | 0.10 | 0.90 |

   c) Dilute each point on the calibration curve 10x; 90µl $ddH_2O$ and 10µl BSA. **Make 3 separate dilutions for each point!**
2. Dilute muscle homogenate (either supernatant or crude) 10x; 90µl $ddH_2O$ and 10µl sample. **Make 3 separate dilutions for each sample!**
3 Dilute 25ml Bio-Rad stock reagent in 75ml $ddH_20$ and filter. Store unused reagent at 4°C.
4. Pipette into the wells of the microtitre plates 10µl of diluted homogenate or standards. Use 10 ml $ddH_2O$ in 6 wells to determine blank values.
5. Using a multi-channel pipette add 200µl of diluted reagent into each well.
6. A microplate reader (Benchmark, Bio-Rad, Hercules, CA) is used for protein content analysis. See next page for instructions.

a. Turn ON (behind machine) and let machine *self diagnose.*
b. Insert your plate of samples carefully. Close the hatch.
c. At start-up screen, press ▶▶
d. Cursor down to ANALY
e. press ▶▶
f. At D/S, Filter, press ▶▶
g. Select SINGLE and set Mes. to 595
h. Hit ENTER
i. Cursor down to MIXING
j. press ▶▶
k. Press YES
l. Cursor down to TIME
m. Using VALUE (123...) key, set to 30 sec
n. Hit ENTER
o. Press START. Discard results.
p. Wait 5 min and press START again.

SAMPLE PREPARATION FOR WESTERN BLOTTING
To ensure consistency between western blots, it is essential to minimize the number of freeze-thaw cycles. Repeated freeze-thaw cycles will affect the quality of the homogenate, thus making it very difficult to keep amount of protein loaded consistent.

**DAY ONE**

**A)** ***Take frozen homogenates out of freezer and turn on heating block to 90 degrees C***

**B)** ***Setting the Separating Gel (10%)***

**STOCK SOLUTIONS**

Separating Buffer:
27.23 g Tris-base (Boehringer Manneheim, #604-205)
80 ml $dH_20$
Adjust pH to 8.8 with HCl. Fill to 150 ml with $dH_20$ and store at 4°C.

Stacking Buffer:
6 g Tris base (Boehringer Manneheim, #604-205)
60 ml $dH_20$
Adjust to pH 6.8 with HCl and fill to 100 ml with $dH_20$.

30% Acrylamide/8% Bis:
87.6 g acrylamide (Gibco BRL, #15512-031)
2.4 g N'N' bis-methylene acrylamide (BioShop, #BIS-001)
Fill to 300 ml with $dH_20$ and store in the dark at 4°C (i.e. wrap in aluminum foil). Re-make fresh each month.

10% Sodium Dodecyl Sulfate:
10 g sodium lauryl sulfate (SDS) (BDH, #B30175)
Add 10 g of SDS and fill to 100 ml with $dH_20$.
Store at room temperature.

Ammonium Persulfate:
0.02 g ammonium persulfate (APS) (Sigma, #A-3578)
Weigh into an Eppendorf. Add 200 µl of $dH_20$. Make fresh daily.

TEMED: Sigma, #T-9281

5 x Tris-GlycineRunning Buffer:
45 g Tris-base (Boehringer Manneheim, #604-205)
216 g glycine (BDH, #B10119)
15 g SDS (BDH, #B30175)
Add 3L $dH_20$. Do not adjust pH.
**WORKING SOLUTION: Need approximately 1000ml per buffer chamber. Dilute 200ml 5x Tris-Glycine Running Buffer with 800ml of $ddH_2O$.

All reagents except AP and TEMED are available from hospital stores. Order Temed (#T-9281) and APS (A-3678) from Sigma.

* For optimal results prepare the gels fresh (i.e. don't store overnight).

1) measure between .02-.03g of APS (solid). Add DDI water to make 10% solution (ie add 200ul of DDI water to .02g APS). Put APS solution in eppendorf tube and put in middle of plastic container.
2) Put together separating gel setup (1.0mm glass plates and green holders). Clean 1.0 mm plates with 70% ethanol. Assemble into the Bio-Rad mini-protean® 3 Cell set-up (Bio-Rad, 165-3301 and 165-33025) as described in the intruction manual. Once set up, pipette in $dH_20$ between the plates and let stand 5-10 min. If the water level drops, there is a leak in the bottom seal, therefore set up apparati again and re-check with water. When the water level is stable after 5-10 min, then drain it, wipe edges with Kim-Wipe, and go to step 3.
3) In 50ml erlynmyer flask, make 10% Separating GEL from recipe in book [DDI water, Separating buffer, SDS, Acrylamide, APS, TEMED].

For example:
To make up 4 1mm thick mini-gels:

| | *7.5% Separating Gel* | *10% Separating Gel* | *4% Stacking Gel* |
|---|---|---|---|
| $DdH_2O$ | 9.7ml | 8.02ml | 6.1ml |
| Separating buffer | 5.0ml | 5.0ml | 2.5ml |
| 10% SDS | 200µl | 200µl | 100µl |
| 30% Acrylamide/ 0.8%Bis | 5.0µl | 6.66ml | 1.33 ml |
| 10% AP Solution | 100µl | 100µl | 50µl |
| Temed | 10µl | 10µl | 10µl |

**Do NOT add AP and Temed until immediately before casting the gel.

## SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

**The gel is an acrylimide polymer with pores in it, and the pore size can be adjusted depending on the molecular weight of the protein of interest (this determines what % separating gel to cast). In general, pore size decreases with increasing % separating gel. Therefore the lower the % of the acrylamide, the easier the transfer will be. For instance if you have a PROTEIN of large molecular weight, you will need to increase the pore size by decreasing % separating GEL. The concentration of the acrylamide needs to be at a level where the protein of interest will migrate at least one-third of the distance from the top of the separating gel to the bottom within the time it takes the dye front to reach the bottom.**

**In the APS-TEMED system, TEMED catalyses the formation of free radicals from persulphate and these in turn initiate polymerization. Increases in either the TEMED or APS concentration increase the rate of polymerization (but beware that excess catalyst can actually inhibit polymerization).**

4) Pipet separating liquid between 1.0mm glass plates, up to bottom of green line (~1cm from top). Fill immediately to the top with DDI water. Leave for 30 mins. to polymerize. After polymerization, a sharp interface between the polymerized gel and the DDI water can be noticed.

### C) *Begin Making Sample Mixtures*

1) Label new eppendorf tube for each subject and place in styrofoam tube holder.
2) Add proper amount of homogenate to newly labeled eppendorf tube (refer to Protein Concentration Table from Bradford Assay).

### D) *Setting the Stacking Gel (4%)*

1) Make 4% stacking liquid from recipe in book [DDI water, acrylimide, stacking buffer, APS, TEMED].
2) Pour water from top of setup (using a kinwipe to remove excess water), and fill to the top with stacking gel liquid.

3) Immediately insert a 1mm 10 well comb (making sure no air bubbles are beneath the comb). Allow gel to polymerize for 30 mins.

***Finish Making Sample Mixtures***

*Ingredients:*

Sample Buffer (SDS Reducing Buffer):

3.55ml $ddH_2O$

1.25ml 0.5M Tris-HCl, pH 6.8 (see below)

2.5ml glycerol

2.0ml SDS (w/v) 10% (see below)

0.5ml Beta-mercaptoethanol

- Mix well; store at 4°C.

Wash (Dilution) Buffer:

2.796g (150mM) KCl

0.6055g (20mM) Tris Base

- Dilute in 250ml of $ddH_2O$; adjust pH to 7.0.

1) Add proper amount of wash buffer (also referred to as dilution buffer) and SDS sample buffer to newly labeled eppendorf tube containing proper amount of homogenate. Ratio of (homogenate + wash buffer): SDS buffer is always 2:1 or 30µl:15µl. Refer to Protein Concentration table from Bradford Assay. For example calculation for 40µg load see below:

*Example:*

| Total [protien] from Bradford | Amount for 40 ug | Dilution buffer | Sample buffer |
|---|---|---|---|
| (a) 1.89 ug/ul | 40ug / 1.89ug/ul = 21.16 ul | 30ul – 21.16ul = 8.84 ul | 15 ul |
| (b) 2.39 ug/ul | 40ug / 2.39 ug/ul = 16.74 ul | 30ul – 16.74 ul = 13.26 ul | 15 ul |

2) Dilute your broad molecular weight standard (BioRad, #161-0319) 1:20 in sample buffer if using AP development.
3) Immediately following the addition of SDS and sample buffer, sample mixtures centrifuged for 10sec. and then placed in the heating block for 7 mins. The biotinylated standards are heated for the last 1-2 minutes only. All samples are placed on ice immediately following the heating.

**The protein mixture in the sample is denatured by heating at 100 degrees C in the presence of excess SDS to polypeptide (at least 3:1). Sodium dodecyl sulfate (SDS) is a 'detergent' that breaks all non-covalent bonds (ie. quarternary, tertiary and secondary protein structure), while Beta-mercaptoethanol (BME) is a 'reducing agent' (or thiol reagent), that breaks disulfide bonds (-S-S-), thus rendering single stranded primary amino acid peptides. SDS is anionic and will bind to the polypeptide in a constant weight ratio (to approximately every 2$^{nd}$ amino acid). The intrinsic charges of the polypeptide are insignificant compared to the negatice charges provided by the bound detergent, so that the SDS-polypeptide complexes have essentially identical charge densities and migrate in polyacrylamide gels of the correct porosity strictly according to polypeptide size.**

F) ***Prepare Running Buffer and Apparatus***

1) Measure 200ml of running buffer (found in fridge in hall) in 1L graduated graduated cylinder. Fill to 1L mark with DDI water.
2) Move prepared gel from green chamber to white chamber (with small glass plates facing inside). Carefully remove the comb. Put white chamber into clamping frame which in turn is placed into the mini tank (clear plastic container).
3) Fill mini tank with running buffer, making sure middle of clamping frame is filled to the brim.
4) In note book, make sure you record which subject samples are to be loaded in which lanes. For instance

| A1 LOW | B1 BROAD |
|---|---|
| 2 F1A | 2 M4A |
| 3 F1B | 3 M4B |
| 4 M1A | 4 M5A |
| 5 M1B | 5 M5B |
| 6 M2A | 6 M9A |
| 7 M2B | 7 M9b |
| 8 M3A | 8 M11A |
| 9 M3B | 9 M11B |
| 10 BROAD | 10 LOW |

5) Using yellow loading guide and loading tips, put 20µl of standards into proper lanes, then 35µl of each subject sample into proper lanes.

Example of loading protocol:

| *Lane* | *Sample* | *Volume (µl)* |
|---|---|---|
| 1 | Molecular weight standard | 20 |
| 2 | S1 Pre training | 35 |
| 3 | S1 Post training | 35 |
| 4 | S2 Pre training | 35 |
| 5 | S2 Post training | 35 |
| 6 | S3 Pre training | 35 |
| 7 | S3 Post training | 35 |
| 8 | S4 Pre training | 35 |
| 9 | S4 Post training | 35 |
| 10 | Molecular weight standard | 20 |

6) Connect assembly to power supply and run gels at 200V until fully migrated (~45 mins). Fully migrated when samples (blue dye) are at bottom edge of plate setup.

**Electrophoresis is an electrical current that runs through the gel and induces the proteins to migrate through the gel toward the anode. Proteins are naturally 'negatively' charged, and the overall charge of the protein in nature is a function of the amino acid sequence, and a very specific property of that protein. However, by coating the protein with SDS, you have, for all intents and purposes, taken this 'natural charge' out of the equation, and thus the proteins will migrate solely as a function of their molecular weight (MW). Small MW proteins will migrate faster, while large MW proteins will migrate slower, and the migration of each individual protein follows a log-linear pattern.**

**The proteins are separated by gel electrophoresis, and transferred to a membrane that binds the proteins nonspecifically. Transfer onto the PVDF membrane is achieved by placing the membrane in direct contact with the gel and then placing the 'sandwich' in an electric field to drive the proteins from the gel onto the membrane.**

G) ***Make Transfer Buffer***

**Western Buffer A:**

30.2g Tris

144g Glycine

- Adjust volume to 1000ml with $ddH_2O$.

Transfer Buffer (1000ml/buffer chamber):

100ml Western Buffer A

10ml 10% SDS

240ml Methanol

- Bring up to 1000ml with dd$H_2O$ and chill.

Add 100ml of Western Buffer A into 1L graduated cylinder. Measure 10ml of 10% SDS solution and add to 1L graduate cylinder containing Western Buffer A. Add 240 ml of methanol to transfer buffer in 1L graduated cylinder (fill to 350ml mark). Fill to 1L with DDI water. Cover with parafilm and mix.

**H)** ***Prepare Transfer Apparatus***

1) Put 2 PVDF membranes (Bio-Rad, #162-0184) into separate Petri dishes and fill halfway with methanol. Let sit for ~10 mins. to open the pores. Then dump out methanol and allow membranes to equilibrate in transfer buffer for ~5 mins.
2) Cut out 4 pieces of filter paper the size of sponges
3) Set up transfer buffer tray on a slant. Black end of gel holder 'sandwich' should be immersed in transfer buffer. All parts of the sandwich should be fully soaked in transfer buffer. It is important to minimize the time the PVFD membranes are not in the transfer buffer.
   Sandwich SET-UP: black end – sponge – filter paper – gel – PVDF membrane - filter paper – sponge – clear end.
4) After the gel has run, dump out the running buffer and mark the LOW molecular weight marker side of the gel by scrapping some of the gel off at the top and the bottom. Low molecular weight marker (and therefore lane 1, is always on the right side of the large 1.0mm glass plate).
5) Fill up mini tank with remaining transfer buffer that is not being used in slanted buffer tray for 'sandwich' set-up.
6) Make the 'sandwich' while keeping it fully submersed in transfer buffer. Place the PVDF membrane on the gel by middle out through carefully holding The two corners of the membrane.
7) After the 'sandwich' is made, immediately transfer to red and black holder already assembled in mini tank with small stir bar and filled with transfer buffer. Remember to put black end of sandwich beside black end of holder.
8) Put mini tank onto tray and then onto stir plate. Put on lid and leave at 100V for 1 hr.

**Western Blotting is the detection of your protein of interest once it has been transferred to a synthetic membrane (in this case the PVDF membrane).**

**I)** ***Make '1 xTBS' solution***

1) Measure 2.42g TRIS.
   29.22g NaCl
   Add these to 1L beaker. Add ~ 900ml of DDI water to beaker. Add HCl until pH is at 7.5. Top to 1L with DDI water and then .....

**J)** ***Make 5% Blocking Solution and Block***

1) Add blocking reagent (non-fat dry milk , bovine serum albumin, or gelatin) to '1 x TBS' (without TWEEN added). Agitate until all blocking reagent is dissolved.
   5% Blocking Solution (5ml per blot):

*Gelatin:*

- Dissolve 1.1 ml 45% Gelatin (Sigma, #G-7765) in 10 ml TTBS.

*Milk:*

- Dissolve 500 mg skim milk (from grocery store) in 10 ml TTBS

***Blocking is done to prevent non-specific antibody binding to other proteins.*

2) Put '1 xTBS' in 2 petri dishes
3) Remove PVFD membrane from 'sandwhich' and mark top corner of Low molecular weight side either A or B. Add PVDF membrane to '1 x TBS' solution at a 45 degree angle. Make sure side of PVDF membrane that was touching the gel is face up when placed in the Petri dish. Place on rocker for 2x5mins.
3) Add blocking solution to 2 petri dishes and then add the PVDF membrane and leave for 1 hr at room temp. or overnight in a refrigerator. During this time the Petri dishes should be placed on a shaker.

**The remaining binding sites on the membrane are blocked to eliminate any further reaction with the membrane.**

**K)** ***Add TWEEN to '1 x TBS' solution***

1) Place '1 x TBS' solution on a stir plate and add 1ml of TWEEN. This will form your TTBS solution.

**TWEEN-20 is essential in washing to eliminate overall background and non-specific hydrophobic reactions. At 0.1% TWEEN will not disrupt binding of primary antibodies to antigens or antigens to the membrane, but will optimize detection sensitivity by eliminating non-specific reactions. Increased concentrations of TWEEN (up to .3%) can be used if background problems persist. However, at higher concentrations (0.3%), TWEEN may affect the reactivity of some antibodies.**

## DAY TWO

### L) *Washing Blots*

1) Pour out blocking solution and fill with TTBS, swirling for 10 seconds. Pour out and repeat 2 more times.
2) Fill Petri dish halfway with TTBS and put on rocker platform for 7 mins. Repeat 2 more times.

### M) Make and *Add Primary Antibody*

1) Add 1g dry milk solution to 10ml TTBS. Add 10µl of primary antibody to give a 1:1000 dilution. Concentration of primary antibody may need to vary. Primary antibody for GLUT 4 is goat anti-human.

1° Antibody Solution (5ml per blot):

- Dilute antibody in TTBS or 1% or 5% blocking solution just prior to use (will have to test which is better for your antibody). Diluting in a blocking solution (a) will help prevent non-specific binding (i.e. block) as well as (b) act as a 'carrier molecule' for the antibody and may increase the chance of the antibody binding to the protein of interest.
- To determine the optimal [antibody], you need to perform a titration. The antibody manufacturer will give you guidelines as to which concentration should be used (e.g. 1:1000-1:2000). Using this as a guideline, you should try to determine the optimal concentration for your conditions. For example, you might want to start with 5 blots: 1:500, 1:1000, 1:1500, 1:2000, 1:2500 and see which gives you the best results.

2) Pour out TTBS solution from Petri dish and split antibody mixture between the two dishes. Set on rocker platform and leave on rocker for 1 hr. (may want to leave overnight).

**A primary antibody that has been raised in some other species (e.g. goat) against your human protein of interest is incubated onto the PVDF membrane. That antibody (e.g. goat anti-human GLUT 4) will bind to the human GLUT 4 with high affinity.**

**N)** ***Washing Blots***

1) Pour out blocking solution and fill with TTBS, swirling for 10 seconds. Pour out and repeat 2 more times.
2) Fill Petri dish halfway with TTBS and put on rocker platform for 7 mins. Repeat 2 more times.

**O)** ***Make and add Secondary Antibody mixture***

1) Mix 3.3µl of both the secondary antibody and the avidin AP conjugate to 10ml of TTBS (1:3000 dilution).
2) Pour out TTBS from Petri dishes and split secondary antibody mixture between them. Set on rocker platform and leave for 1hr.

**A secondary antibody that has been raised in some other animal (e.g. rabbit) against your primary antibody is then incubated onto the PVDF membrane. The secondary antibody (e.g. rabbit anti-goat GLUT 4) will bind to goat GLUT 4 (which is your primary antibody) with high affinity. The secondary antibody is tagged with alkaline phosphatase (AP) or horse radish peroxidase (HRP). This enzyme is used to activate a chemiluminescent reagent in proportion to the amount of protein on the membrane. The membrane can then be exposed to Kodak film, developed, and analyzed using densitometry**.

**P)** ***Washing Blots***

1) Pour out blocking solution and fill with TTBS, swirling for 10 seconds. Pour out and repeat 2 more times.
2) Fill Petri dish halfway with TTBS and put on rocker platform for 7 mins. Repeat 2 more times.

**Q)** ***Dark Room***

1) Collect things needed:
   a) dark room key
   b) yellow tape to put over key hole
   c) 2500ml adjustable pipet and pipet tip
   d) timer
   e) orange light box
   f) metal pen (scrapper)
   g) immunostar substrate
   h) tweezers
   i) saran wrap
   j) black sharpee marker
   k) ruler
   l) film cassette bag
   m) block of lead
   n) glass plates
   o) 2 petri dish tops
   p) film (in yellow Kodak box)
   q) paper towel
   r) kim wipes

2) Set up dark room
3) Place tape over key hole and lock door. Cover bottom crack of door with green cover. Turn off lights and prepare film. Divide it into 3 sections by making 2

lines with the marker (at 8.5cm and 17cm). Divide the film into 2 sides, A and B. Finally mark the corner of each section by writing the time of exposure (usually 30, 45, 60 sec.). Load prepared film into film holder and turn the lights back on.

4) Put 2.5ml of immunostar substrate into each Petri dish and add blots for 5 mins. Blots should be face up. Add blots by sliding in one side first. Make sure blots are fully covered then do not touch.
5) Remove blots from immunstar substrate and place on saran wrap face down. Make sure non air bubbles are under saran wrap. Place down by middle-out technique. Cover with saran wrap.
6) With lights off, place film on top of blots for designated time using glass plates.

**R)** ***Developing***

1) Take film to developing room on 2nd floor hospital and cross your fingers.

**S)** ***Band Density Determination***

Blots can be digitized using a scanner or the Epi –Illumination UV Darkroom – be sure that ALL the setting are identical for each blot digitized within a study. A computerized image analysis system *(LabWorks, UVP Laboratory products, Upland, CA)* is used to determine respective band density.

For densitometry in Labworks:

(a) Open image
(b) Under TOOLS bar, select *Area Density*
(c) Select *Intensity Calibration*
(d) Press NEW
(e) Select *Standard Optical Density*
(f) OK
(g) Select *Define Region*
(h) Draw around your areas of interest. When finished, select END.
(i) *Total Optical Density* can be exported to Excel, if a spreadsheet is open.
    a. Select DDE options. Set columns and rows to fit your spreadsheet.
    b. Select DDE to excel.

Because of the high variability between blots, only within blot comparisons can be made. One cannot compare between blots. Therefore, be sure to load all samples for the same subject on the same blot (e.g. PRE and POST some intervention). Usually you will express the % change from pre, and graph it normalized to 1 (i.e. PRE value set to 1).

# APPENDIX 12

CITRATE SYNTHASE ACTIVITY ASSAY

## Citrate Synthase Activity Assay

### Part A. Homogenization Protocol:

1) Make homogenizing buffer. Ingredients: a) 0.340g (5mM) potassium dihydrogen orthophosphate ($KH_2PO_4$)
   b) 0.186g (1mM) ethylene diamine tetraacetic acid (EDTA)
   c) 0.008g (0.1mM) dithiothreitol (DTT)

   Add ingredients with 450ml of $ddH_2O$. pH to 7.4 and fill to 500ml with $ddH_2O$.

2) Weigh and record 15-25mg of muscle from each subject (pre and post training).
3) Add 425µl of homogenizing buffer to the above muscle sample. Grind 50 times on ice and then transfer to Eppendorf tubes. Place 50µl of homogenate (buffer + muscle) into approximately 9 different Eppendorf tubes to avoid "freeze-rethaw" cycles.

### Part B. Assay Solution Preparation:

1) 100mM Tris buffer: add 6.06g of Tris base (Boehringer Manneheim 604205) and fill to 400ml with $ddH_2O$. pH to 8.0 and fill to a final volume of 500ml. Stable for 3 months if stored at 0°-4°C.
2) 4 mg/ml DTNB [5,5' – dithiobis(2 – nitrobenzoic acid); Sigma D8130]: add 4mg of DTNB to 1ml of Tris buffer.
3) 6.6 mg/ml OAA (oxaloacetate; Sigma O4126): add 6.6mg of OAA and 1ml of Tris buffer.
4) 30mM acetyl CoA (acetyl coenzyme A; Sigma A2056): to a 10mg bottle add 402.8µl of $ddH_2O$.

### Part C. Assay Protocol:

The reaction involves the measurement of citrate synthase activity by linking the release of CoASH to the colormetric agent DTNB 5,5-dithiobis-2-nitrobenzoate. Changes in absorbance are followed at 412nm by a UV spectrophotometer. The limited pH range for this assay is 7.4-9.0.

Oxaloacetate + acetyl CoA + $H_2O$ --------***citrate synthase***--------> CoASH + Citrate + $H^+$

CoASH + DTNB $_{\text{pale yellow}}$ ----------> CoA + DTNB $_{\text{dark yellow}}$

The assay is prepared in a clear plastic cuvette that is placed into the spectrophotometer. Each assay reagent, including the blanks, were performed in duplicate (2 cuvette tubes).

1) Heat a portion of the Tris buffer to 37°C in a water bath. Add 1ml of the warm Tris buffer to the cuvette.
2) Add 10µl of DTNB to the cuvette.
3) Add 2µl of acetyl CoA to the cuvette.
4) Add 10µl of muscle homogenate to the cuvette. Place the cuvette in the spectrophotometer.
5) Zero the spectrophotometer and makes sure it is set to read the absorbance every 30 seconds.
6) Add 10µl OAA to the cuvette. This initiates the reaction. Quickly mix the solutions in the cuvette, and then start the spectrophotometer reading.
7) Do not record the absorbances during the first minute. Allow the reaction to become stable during this time.
8) Use the change in absorbance from 1 to 3 minutes for calculating CS activity.

Part D. Calculation of Citrate Synthase Activity:

**Enzyme Activity = (OD/(ε x $10^{-6}$)) (dilution) (volume of homogenizing buffer used to prepare muscle/ wet weight of the muscle) ÷ 1000**

OD = (absorbance at 3 min – absorbance at 1 min) / 2 min
ε = the molar extinction coefficient for DTNB at 412nm = 13,600
Dilution = (volume of Tris buffer / homogenate added) = (1ml / 10µl)
Volume of homogenizing buffer used to prepare muscle = 450µl

The enzyme activity was calculated and reported in micromoles per minute per gram of muscle wet weight.

**Units = µmol/ min/ g w.w.**

# APPENDIX 13

## CAPILLARY STAIN

## α-AMYLASE PERIODIC ACID SCHIFF STAIN

## Amylase Periodic Acid Schiff Stain

Prior to the PAS stain, α-amylase is used to hydrolyze the glycogen in the cell. When the PAS stain is used on these muscle sections, the Schiff's reagent can react with the mucoproteins and glycoproteins of the capillary membrane, which remain intact. Normally the dark PAS staining for glycogen obscures the fact that capillary endothelium and muscles membranes stain with PAS. Removal of the glycogen first, allows these features to be identified.

***Part A***

**REAGENTS:**

**I. Schiff's reagent:**

- Basic Fuschin, also called Pararosaniline (Sigma P7632 Base). **1.0g**
- 1.0 N HCl (molarity=12.1). ∴ to make a 1.0 N HCl solution, dilute every 1ml HCl into 12.1ml dd$H_2O$. **20.0 ml**
- Sodium metabisulfite ($Na_2S_2O_5$) (Sigma S 1516). **1.0g**
- Activated charcoal (Sigma C 4386). **2.0g**
- Distilled $H_2O$. **210.0 ml**

A) Boil 210.0 ml of dd$H_20$ in glass beaker covered on top with tin foil and placed on hotplate. As soon as it boils (but make sure it is bubbling), add 1.0g of basic fuschin. Stir with a small stiring bar for 5 minutes.
B) Cool to 50°C (use a glass thermometer) and filter using a coffee filter in funnel. Poor into new 500ml glass erlynmeyer-type beaker, through the coffee filter/funnel apparatus.
C) Add 20.0 ml of1.0 N HCl to the filtrate and allow to cool to room temperature.
D) After solution cools to room temperature, add 1.0g of sodium metabisulfite. Put a rubber stopper in the top of the erlynmyer beaker and cover with entire beaker with tin foil and store in the dark overnight.
E) Add 2.0g of charcoal. Stir for approximately 10 minutes using a stir bar. Filter into new glass beaker using the coffee filter/funnel apparatus. The filtrate should be colourless. Store in the dark (wrap in tin foil) at 0-4°C for future use. As sson as a pinkish colour develops, discard. Allow to warm to room temperature before using.

## II. PAS Fixative:

- Absolute alcohol **16.0 ml**
- Chloroform **3.0 ml**
- Glacial acetic acid **1.0 ml**

** must make this fresh daily.

## III. Periodic Acid:

- Periodic acid (Sigma P5463) **0.24g**
- Distilled $H_2O$ **20.0 ml**

**must make this fresh daily.

## IV. Amylase solution:

- ***this can be played with depending on the muscle tissue section you are staining. I found what worked for some tissues, didn't for others. Older protocols call for a 1% Amylase solution, but I found that generally a more concentrated solution stained better. My suggestion is to start with a 3% Amylase solution, but increase or decrease the concentration depending on the intensity (dark or not dark) of your resulting stain:
- Amylase (Sigma A 6255) **600mg**
- Distilled $H_2O$ **20 ml**

*Part B*

**PROTOCOL:**

1) Use 10μm tissue muscle sections.
2) Place in PAS fixative for 7 min. at room temperature.
3) Rinse approx. 7x in $ddH_2O$ by dunking lightly into water.
4) Incubate at 37°C, shaking lightly, for 40-45 min. in Amylase solution (***the time can be played with here as well, depending on your concentration of amylase. The stronger your amylase concentration, generally the less time required in the incubation. For a 3% amylase solution, I suggest approximately 40 min.). **The amylase solution hydrolyzes the glycogen in the cell.**
5) Repeat $ddH_2O$ rinse.
6) Place in Periodic acid solution for 12-15 minutes at room temperature. **The periodic acid splits the adjacent alcohol groups forming aldehydes.**
7) Repeat $ddH_2O$ rinse.
8) Place in Schiff's Reagent in shaker at 37°C for 10 minutes (often only a few minutes is enough for the stain to develop, but I found that the stain doesn't tend to increase in intensity if left in for the full 10 minutes). **The aldehydes react with Schiff's solution to produce a pink stain.**
9) Place in cold tap water for 10 light dunks.
   Dehydrate using 75%, 90%, 100% ethanol as usual.