UNIVERSITY OF ALBERTA

Effects of Anabolic Steroids and Electrical Stimulation on Collagen Production in Traumatized Rat Muscle

BY

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science.

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Dedication

To MOM and DAD: Mary-Claire and Ubald, who have been so understanding and supportive throughout my education.

To JOAN: who gave me the understanding and support only a true friend could give.

Abstract

The effects of an anabolic steroid (Decadurabolin) and electrical muscle stimulation on the collagen and non-collagenous protein contents of skeletal muscle of the lower limb during recovery from acute blunt trauma was investigated. Thirty-seven male Sprague-Dawley rats were trained following a common seven week endurance training program, producing a significant ($p<0.05$) increase in citrate synthase activity. Rats of the same weight were then randomly assigned to one of the five groups following the seven weeks of training: 1) No Trauma; 2) Trauma, untreated; 3) Trauma, treatment with anabolic steroid; 4) Trauma, treatment with electrical stimulation; 5) Trauma, treatment with anabolic steroid and electrical stimulation. Muscle trauma was achieved utilizing a humane and reproducible experimental device. After ten days of treatment, results indicated that rats in group 3 produced more collagen than groups 1,2 and 4 ($p<0.05$). In addition, the contralateral uninjured lower limb muscles of rats in group 3 formed more collagen than did the corresponding muscles of rats in groups 4 and 5 ($p<0.05$). The amount of non-collagenous protein in traumatized lower limb muscles was significantly lower ($p<0.05$) in group 5 compared to groups 2 and 4. The results suggest that a therapeutic dosage of anabolic steroid significantly increases the production of collagen in traumatized skeletal muscle and may be detrimental to the muscle's complete regeneration.

collagen, protein, anabolic steroid, electrical stimulation, skeletal muscle, trauma, citrate synthase, exercise.

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Table of Contents

		Page
Chapter 1	Introduction	1
	Statement of the problem	2
	Purpose of the study	3
	Research Hypotheses	4
	Delimitations	7
	Limitations	8
	Definition of terms	9
Chapter 2	Literature Review	
	Muscle	10
	Injury	10
	Exercise	16
	Summary	18
	Anabolic Steroids	19
	Structure and Binding	19
	Effects	26
	Summary	28
	Electrical Stimulation	29
	Currents	30
	Effects	32
	Summary	36
Chapter 3	Methods and Procedures	37
	Animals	37
	Exercise Training Program	37
	Tissue Trauma	38
	Tissue Treatment	40
	Tissue Sampling	41
	Statistical Analysis	42
Chapter 4	Results	43
	Training Effect	43
	Tissue Treatment	43
	Summary	56

		Page
Chapter 5	Discussion	63
	Training Effect	63
	Tissue Treatment	64
	A) Anabolic Steroids	66
	B) Electrical Stimulation	69
	C) Anabolic Steroids and Electrical Stimulation	71
	Summary	72
Chapter 6	Summary and Conclusions	74
	Conclusions	75
	Clinical Significance	78
References for Introduction		79
References for Literature Review		
Muscle		82
Anabolic Steroids		88
Electrical Stimulation		93
References for Methods and Procedures		98
References for Discussion		99
Appendix 1	Anabolic Steroid Injection Protocol for a Rat	105
Appendix 2	Electrical Stimulation Protocol for a Rat	106
Appendix 3	Biochemical Analysis	
	Method of Woessner	107
	Lowry's Non-Collagenous Protein Determination	108
	Citrate Synthase Assay	109
Appendix 4	Training Protocol	112
Appendix 5	Current Output from Electrical Stimulation	113

List of Tables

Table	Description	Page
1	Effects of Training on Citrate Synthase.	44
2	Effects of Trauma and Anabolic Steroids on Non-Collagenous Protein Content 10 Days Post-Injury.	46
3	Effects of Trauma and Anabolic Steroids on Collagen Content 10 Days Post-Injury.	46
4	Effects of Trauma and Electrical Stimulation on Non-Collagenous Protein Content 10 Days Post-Injury.	47
5	Effects of Trauma and Electrical Stimulation on Collagen Content 10 Days Post-Injury.	47
6	Effects of Trauma, Anabolic Steroids and Electrical Stimulation on Non-Collagenous Protein Content 10 Days Post-Injury.	49
7	Effects of Trauma, Anabolic Steroids and Electrical Stimulation on Collagen Content 10 Days Post-Injury.	51
8	Effects of Trauma on Non-Collagenous Protein Content 10 Days Post-Injury.	52
9	Effects of Trauma on Collagen Content 10 Days Post-Injury.	52
10	Effects of Anabolic Steroids and Electrical Stimulation on Non-Collagenous Protein Content 10 Days Post-Injury.	54
11	Effects of Anabolic Steroids and Electrical Stimulation on Collagen Content 10 Days Post-Injury.	54

Table	Description	Page
12	Matrix of Significant Difference in the Traumatized Leg Between Control, Trauma, Treatment with Anabolic Steroids, Treatment with Electrical Stimulation and Treatment with Anabolic Steroids and Electrical Stimulation for Non-Collagenous Protein Content.	57
13	Matrix of Significant Difference in the Non-Traumatized Leg Between Control, Trauma, Treatment with Anabolic Steroids, Treatment with Electrical Stimulation and Treatment with Anabolic Steroids and Electrical Stimulation for Non-Collagenous Protein Content.	59
14	Matrix of Significant Difference in the Traumatized Leg Between Control, Trauma, Treatment with Anabolic Steroids, Treatment with Electrical Stimulation and Treatment with Anabolic Steroids and Electrical Stimulation for Collagen Content.	60
15	Matrix of Significant Difference in the Non-Traumatized Leg Between Control, Trauma, Treatment with Anabolic Steroids, Treatment with Electrical Stimulation and Treatment with Anabolic Steroids and Electrical Stimulation for Collagen Content.	62
16	Current output of Focus NMS unit.	114

List of Figures

Figure	description	Page
1	Structure of Testosterone Hormone	20
2	Structure of Stanozolol, 17a-alkylated Synthetic Anabolic Steroid.	21
3	Structure of Nandrolone Decanoate (Decadurabolin), 17b-estrified Synthetic Anabolic Steroid.	22
4	Research Design.	39
5	Histogram of the Effects of Trauma, Anabolic Steroids and Electrical Stimulation on Non-Collagenous Protein Content 10 Days Post-Injury.	58
6	Histogram of the Effects of Trauma, Anabolic Steroids and Electrical Stimulation on Collagen Content 10 Days Post-Injury.	61
7	Circuit testing current output of Focus NMS unit.	113

Chapter 1

Introduction

Muscle trauma occurs daily in the life of an athlete. It has been reported that strenuous exercise of an insufficiently trained muscle liberates excess quantities of metabolites and potentially increases cell damage which manifests as aching, pain and swelling of the muscle and delayed muscle soreness (4,26).

The healing process of the traumatized muscle progresses rapidly when ideal conditions are present. Electrical stimulation of the skeletal muscle can be used to facilitate muscle contraction, inhibit pain, and decrease spasticity and spasm (11,25). In addition, electrical stimulation has been used as a mechanism for augmenting muscle strength and enhancing local circulation in contracting muscle (10). Bourguignon and Bourguignon (10) suggest that the rates of both protein and DNA synthesis were significantly increased by specific combinations of high-voltage and pulsed galvanic stimulation voltages.

Anabolic steroids potentially enhance the muscular healing process, because of their muscle building characteristics. Androgens are steroid hormones that possess virilizing (androgenic) actions and consequently, serve to stimulate differentiation and maintenance of the androgenic sensitive tissues . These hormones also play an important role in facilitating protein synthesis (anabolic actions) in androgen-sensitive tissues such as skeletal muscle, kidney, and bone. Michna (23) demonstrated that the duration of

anabolic steroid treatment affected the number of developing collagen fibrils in muscle and tendon. During the first week of steroid treatment, the extracellular accumulation of collagen increased, suggesting increased synthesis. The number of collagen fibrils and their volume-density also increased. This increase is an adaptation of the tendon to the increased muscle strength. However, after ten weeks of treatment, the number of high volume-density fibrils decreased and the number of low volume-density fibrils increased significantly. This variation in fibre size suggests that tendon strength per unit area may decrease with long-term treatment with anabolic steroids.

The therapeutic effects of electrical stimulation and anabolic steroids on muscle healing after blunt trauma may be examined by measuring the hydroxyproline content of muscle in the rat blunt trauma model (15).

Although much is known about the applications of electrical stimulation and anabolic steroids on both animals and humans (1,2,6,9,12,14), additional research is required to clarify the mechanisms of actions and physiological effects of these therapeutic modalities.

STATEMENT OF THE PROBLEM

Considerable research has been reported in the areas of electrical stimulation (1,2,6,7,8) and anabolic steroids (9,12,14,16,19). None of the sources have focused on the trained skeletal muscle and the therapeutic effects of these modalities on collagen

production with adequate applications of dosage.

A better understanding of the physiological effects of the application of both these modalities is needed, especially concerning their effects on collagen and non-collagenous protein production in the injured subjects.

In the case of anabolic steroids, the present study was designed to evaluate whether a therapeutic dosage of Decadurabolin used alone or in conjunction with electrical stimulation would have a physiological effect that might potentially increase the speed of recovery of the injured skeletal muscle.

Electrical stimulation has been known to increase strength in the muscle through implanted electrodes (8). No previous study utilizing the animal model has examined the effect of electrical stimulation when applied to the skin of the animal such as done with humans during the rehabilitation of a muscular injury in the clinical setting (13,22). It was thus deemed necessary to use surface electrodes during this study.

PURPOSE OF THE STUDY

The purpose of this study was to:

a) Determine if anabolic steroids and/or electrical stimulation had an effect on collagen and non-collagenous protein production in exercised skeletal rat muscle after trauma.

b) Determine which of the following therapeutic modalities, electrical stimulation or anabolic steroids or both, had a greater effect on collagen and non-collagenous protein production in exercised skeletal rat muscle after trauma.

RESEARCH HYPOTHESES

The following hypotheses were tested in this study:

1) There is no significant difference between the citrate synthase levels in the non-exercised non-traumatized muscle and the exercised non-traumatized muscle.

2) There is no significant difference between the collagen content in the exercised traumatized muscle untreated and the exercised traumatized muscle treated with anabolic steroids.

3) There is no significant difference between the non-collagenous protein content in the exercised traumatized muscle untreated and the exercised traumatized muscle treated with anabolic steroids.

4) There is no significant difference between the collagen content in the exercised traumatized muscle untreated and the exercised traumatized muscle treated with electrical stimulation.

5) There is no significant difference between the non-collagenous protein content in the exercised traumatized muscle untreated and the exercised traumatized muscle treated with electrical stimulation.

6) There is no significant difference between the collagen content in the exercised traumatized muscle untreated and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

7) There is no significant difference between the non-collagenous protein content in the exercised traumatized muscle untreated and the exercised traumatized muscle treater with anabolic steroids and electrical stimulation.

8) There is no significant difference between the collagen content in the exercised traumatized muscle treated with anabolic steroids and the exercised traumatized muscle treated with electrical stimulation.

9) There is no significant difference between the non-collagenous protein content in the exercised traumatized muscle treated with anabolic steroids and the exercised traumatized muscle treated with electrical stimulation.

10) There is no significant difference between the collagen content in the exercised traumatized muscle treated with anabolic steroids and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

11) There is no significant difference between the non-collagenous protein content in the exercised traumatized muscle treated with anabolic steroids and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

12) There is no significant difference between the collagen content in the exercised traumatized muscle treated with electrical stimulation and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

13) There is no significant difference between the non-collagenous protein content in the exercised traumatized muscle treated with electrical stimulation and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

14) There is no significant difference between the collagen content in the exercised non-traumatized muscle untreated and the exercised traumatized muscle untreated.

15) There is no significant difference between the non-collagenous protein content in the exercised non-traumatized muscle untreated and the exercised traumatized muscle untreated.

16) There is no significant difference between the collagen content in the exercised muscle and the exercised traumatized muscle treated with anabolic steroids.

17) There is no significant difference between the non-collagenous protein content in the exercised muscle and the exercised traumatized muscle treated with anabolic steroids.

18) There is no significant difference between the collagen content in the exercised muscle and the exercised traumatized muscle treated with electrical stimulation.

19) There is no significant difference between the non-collagenous protein content in the exercised muscle and the exercised traumatized muscle treated with electrical stimulation.

20) There is no significant difference between the collagen content in the exercised muscle and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

21) There is no significant difference between the non-collagenous protein content in the exercised muscle and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

DELIMITATIONS

The following delimitations applied to this study:

1- Only 37 male Sprague-Dawley rats between the weights of 180-200g were used.

2- Only one established training protocol was used. (See Appendix 4.)

3- A Focus NMS unit was used as the electrical stimulator. (ANSI/AAMI NS4-1985).

4- The calibration of the Focus NMS unit was standardized. (ANSI/AAMI NS4-1985).(See Appendix 5.)

5- For the electrical stimulator, two surface electrodes of equal size and constant placement were used.

6- The setting of the electrical stimulator at 50 pulses per second, 5 seconds on and 5 seconds off, with a monophasic wave.

7- The pre-set amount (5mg) of injectable Decadurabolin was used as the anabolic steroid.

8- The amount of force delivered by the trauma device.

LIMITATIONS

The following limitations applied to this study:

1- The inability to control the rats daily activity outside of the training protocol.

2- The inability to ensure that each rat obtained the same training effect from the established protocol.

3- The inability to assure that the blunt trauma was of the same force for the various rat muscle sizes.

4- The daily variation in the skin tolerance (impedance) of the rat during electrical stimulation.

DEFINITION OF TERMS

The following are defined as they were utilized in this study:

Anabolic steroid: A synthetic derivative of testosterone which mimics preferentially the anabolic and to some degree, the androgenic properties of testosterone (14,18,20).

Collagen: The major protein of the white fibres of connective tissue, cartilage and bone. It is high in glycine, alanine, proline and hydroxyproline (28).

Electrical stimulation: An artificial stimulation of a muscle or motor nerve affecting a minimum twitch produced by the passage of an electrical current applied topically over the motor point and muscle belly causing a contraction of the muscle (17,25).

Hydroxyproline: An amino acid found among the hydrolysis products of collagen; not found in proteins other than those of connective tissue.(28).

Therapeutic dosage: The recommended regimen governing the size, frequency and amount of an agent pertaining to medical treatment to be administered to a patient (24).

Chapter 2

Review of literature

MUSCLE

A) Injury

Approximately 40 per cent of the body is skeletal muscle (23). All skeletal muscles are composed of numerous fibres ranging between 10 and 80 microns in diameter. In most muscles the fibres extend the entire length of the muscle, and except for about 2 per cent of the fibres, each is innervated by only one nerve ending, located near the middle of the fibre (23).

One of the fundamental manifestations of disease and/or injury to the skeletal muscle is a variation in fibre size, which frequently is due to atrophy of some of the muscle fibres. For example, prolonged bed rest or immobilization, which does not allow the muscle to engage in sufficient activity, may engender disuse atrophy (26).

Muscle trauma occurs in all types of activities. In sports, its frequency increases because of prolonged activity, intense training, over-stretching and unintentional or intentional direct contact. The most common mechanism is elongation (stretch) or blunt trauma. To effectively evaluate different treatments of muscle trauma, a standard injury model with respect to size and location is essential. Blunt trauma (19) to the hind limb of the animal model is therefore frequently used to create an artificial injury. Muscle

ruptures in athletes are not usually associated with skin tears; therefore the model should ideally leave the skin intact (29).

Muscle cells are composed mainly of proteins. Proteins are long chains of amino acids with different functions that depend not only on the specific sequence of amino acids (primary structure), but also on secondary structure or side-chain and on the overall configuration of the protein molecule. There are thousands of important proteins which have certain general functions such as transport, storage, motion, structure, growth and hormonal activities (22). Collagen falls into the structure category because it provides a framework of intercellular tissue support in connective tissue, cartilage, bone and other tissues (22). It provides strength and confers form while allowing flexibility (36,46). This protein is composed of linear, unbranching sequences of 20 or so amino acids. An excellent way of recognizing collagen is by its amino acid composition i.e. the relative frequencies of the different amino acid residues (46).

The two main amino acid constituents of collagen which are used to establish its content in muscle are glycine and hydroxyproline. Glycine comprises approximately one third of all residues. Proline is a derivative of hydroxyproline, which is not a naturally occurring free amino acid. The foregoing chemical change takes place after the protein is synthesized. Hydroxyproline is found only rarely in other proteins but it comprises about 10% of all the amino acids in collagen while a further 10% exists as unconverted proline (31,46).

Normal metabolism involves both the synthesis of proteins and their degradation by hydrolysis. The breakdown of a protein into its constituent amino acids is

accelerated, like most other chemical reactions in the body, by enzymes, specifically, by proteases (46). Laurent (27) stated that muscle growth requires the degradation of both the contractile proteins of muscle; and of collagen the major extracelluar connective tissue component (27).

Accelerated net protein breakdown and negative nitrogen balance are characteristic of the altered metabolism that attends trauma (18). Proteolysis in skeletal muscle is a major source of increased urinary nitrogen loss. Although directly injured tissues contribute to nitrogen wasting, a major systemic catabolic response occurs in uninjured muscle as well. In the uninjured muscle, a progressive metabolic adaptation occurs in proportion to the rise in metabolic rate (34).

Collagen turnover in injured muscle is quite rapid. According to a study by McAnulty and Laurent (32), the turnover time could be as short as 15 minutes, which is much more rapid than traditionally believed.

In the healing process of skeletal muscle after injury, two competitive events occur simultaneously namely; 1) the regeneration of disrupted muscle fibres and 2) the production of a connective tissue scar. The latter is capable of inhibiting the complete regeneration of the muscle (31). The local response to injury includes stimulation of cell proliferation and the synthesis of extracelluar matrix components (30,31). During the formation and maturation of granulation tissue, fibroblasts are active in synthesizing collagen (8,29). After an acute inflammatory reaction, collagen is gradually reabsorbed, but if the inflammatory reaction persists, the amount of collagen remains high, and in time leads to fibrosis (11,31).

In the early stages of muscle healing and granulation tissue formation, there is an increase in the synthesis of Type III collagen (7,16,20). As the granulation tissue matures, it is replaced by predominantly Type I collagen (20,30). It has been suggested that the low tensile strength of granulation and scar tissues is related to the high proportion of Type III collagen present in the early stages of healing (21). In all tissues, collagen fibres provide mechanical support, and as such must be highly cross-linked to form an inextensible, stress-bearing fibre (10).

Collagen is thought to be necessary for muscle regeneration, forming a sheath around fusing myoblasts during myotube formation (1,9). However, when a large volume of muscle is devitalised by major trauma, the proliferation of fibroblasts rapidly can lead to the formation of excessive scar tissue, which may form a dense mechanical barrier to the regenerating muscle fibres (33).

The regeneration process in muscle occurs with the help of satellite cells. Satellite cells, which are the myogenic stem cells of mammalian skeletal muscle, are activated upon muscle injury and participate in the repair or replacement of damaged muscle fibres (38). Morphologically, satellite cells are a population of small, flattened mononucleate cells which lie in close apposition to the surface membrane of the striated muscle fibre, just beneath the basal lamina (15). These satellite cells appear to be evenly distributed along the length of the myofibres (37), but are not entirely random, in location as they are found more frequently in close association with intrinsic myonuclei than would occur by chance (37). The satellite cells also commonly occur in increased numbers adjacent to the sole plates of neuromuscular junctions.

Increased numbers of satellite cells are also found in association with the polar intracapsular regions of intrafusal myofibres in muscle spindles. As already indicated, satellite cells are considered to be a persisting population of myoblastic stem cells, which are the source of additional myofibre nuclei during the hypertrophic phase of muscle growth. The satellite cells are also the source of the cells responsible for the regenerative repair of damaged muscle fibres in situ and in muscle cells cultured in vitro (12). Satellite cells divide continuously throughout the period of active myogenesis (24). With the cessation of growth, satellite cell numbers decline to about 5% and 2% of the total number of myofibre nuclei in small animals and in man respectively (34).

Two types of regeneration occur after segmental necrosis. In continuous regeneration, or "budding", the regeneration begins at the healthy end of a severed fibre. Sarcolemmal nuclei migrate into the junctional zone, and myoblasts that have migrated outside the fibre may fuse with it (45). In many instances, the regenerative process results in a series of muscle sprouts, sometimes forming a compact cluster of five or more, which, in cross-section, fit together. The parent fibre from which they originate, is usually becomes enlarged (45).

In discontinuous or embryonal regeneration, mononuclear myoblasts lying free in the tissue align in a single row and then fuse to form a myotube, which will grow and mature into a fibre of adult type, reminiscent of myogenesis in fetal life (40).

Proximal to an injured region, sarcolemmal nuclei with some surrounding cytoplasm become sequestered within a membrane from the remainder of the fibre, and appear as satellite cells. The rapid division of satellite cells results in daughter cells

which develop into myoblasts; the latter are capable of migrating outside the fibre (39,45).

The effectiveness of regeneration depends upon whether or not the scaffolding of the basement membrane and supporting tissues remains intact, and requires that the accompanying inflammation subsides. Preservation of the basement membrane is required for isomorphic regeneration, in which the orientation of regenerating fibres becomes parallel to the original fibres (45). The sarcolemma usually disappears over necrotic segments. Its destruction probably facilitates the migration of phagocytes into the softened, necrotic muscle fibre, and of satellite cells out of the same fibre (45). The removal of dead tissue by macrophages is a requirement for repair.

It is important to recall that in work by Shultz (39), there appeared to be no recruitment of myogenic cells from adjacent muscle fibres. The situation is quite different within a muscle fibre where satellite cells migrate for relatively long distances to participate in the repair of the site of damage (38). Turpin (43) investigated the relative contribution of satellite cells and myotubes from neonatal rat muscle during myogenesis in vitro. His study confirmed the results of previous in vivo autoradiographic studies, which indicated that satellite cells are the only significant source of regenerating myoblasts.

The sarcolemma is the cell membrane of the muscle fibre. However, the sarcolemma consists of a true cell membrane called the plasma membrane, and an outer basement membrane coat, consisting of a thin layer of polysaccharide material containing numerous thin collagen fibrillae. At the end of the muscle fibre, this surface layer fuses

with a tendon fibre (23).

The myofibrils are suspended inside the muscle fibre in a cytoplasmic matrix called sarcoplasm, which is composed of intracellular constituents. The fluid of the sarcoplasm contains large quantities of potassium, magnesium, phosphate, and protein enzymes. Also present are large numbers of mitochondria (23).

B) Exercise

Mammalian skeletal muscle is composed of different muscle fibre types, which are distinguished on the basis of their physiological and biochemical characteristics (17). In the rat, for example, a distinction can be made between fast and relatively slow contracting fibres. The fast contracting fibres are further distinguished by their oxidative capacity. Thus, there are fast-twitch red (highest oxidative capacity), a fast twitch white (lowest oxidative capacity), as well as slow-twitch red (intermediate oxidative capacity) skeletal muscle fibre types (42). The fast twitch red fibres are thought to be the principle muscle fibres involved during mild to moderate physical activity that can be maintained for prolonged periods (3). These fibres enjoy a rich blood supply (35) and are relatively resistant to fatigue (42). On the other hand, the low oxidative white fibres which have a relatively high glycolytic capacity (42) are poorly perfused with blood (35) and fatigue rapidly (42). Therefore, the latter fibre type should be recruited sparingly, and only when essential to enhance the performance of the entire muscle (42).

All three types of skeletal muscle fibres adapt to endurance exercise by an increase in respiratory capacity (4). In rats, a program of prolonged running can induce

as much as a twofold increase in the levels of activity of a number of mitochondrial enzymes in skeletal muscle (5,28). Citrate synthase is a respiratory enzyme which is commonly used as marker for a "training effect" (6).

Savolainen (36) showed that the biosynthesis of collagen in muscle decreases during reduced muscular activity. On the other hand, the biosynthesis of collagen in connective tissue is accelerated by training (41) and during compensatory muscular hypertrophy (44). Eccentric and weight-bearing components of training cause muscular damage (2). After strenuous exercise the increased collagen synthesis seems to be part of the regenerative process which follows exercise-induced muscular injury (25).

Along similar lines, Karpakka (25) indicated that training causes an increase in oxidative enzymes and in the quantity of hydroxyproline. On the other hand, immobilization causes a decrease in oxidative enzymes as well as in the quantity of hydroxyproline. The key finding from his study is that the decrease in both oxidative enzyme capacity and hydroxyproline is less in individuals who were trained prior to immobilization, as opposed to untrained individuals who were immobilized (25).

The duration of each individual exercise bout is an important determinant of the adaptive response (13). Although longer exercise bouts bring about larger responses, there appears to be an upper exercise duration wherein no further increase in oxidative capacity is established (44). The duration of the training program is also important, since the actual increase in mitochondrial content depends on the length of the training (25). This influence is characterized by a first-order process and is dependant on the turn-over rate of the mitochondrial proteins (14). Thus it is important to maintain a training

program for a sufficient length of time to permit the cellular adaptation to fully develop (25).

C) Summary

Following injury, the muscle fibre reacts in many respects as do other living cells in the body. However, the range of structural alterations in muscle is greater than is seen in most other cell types in the body. There are a number of modifications that are unique because of the special features of muscle histology and physiology. It should be realized that the structural changes seen in damaged muscle fibres are associated with more fundamental abnormalities, which only can be revealed by molecular, biological, biochemical or immunological methods (45).

ANABOLIC STEROIDS

Self-administration of anabolic steroids to increase muscular strength and lean body mass is a widespread practice among athletes, even though the indiscriminate use of such drugs may constitute a serious risk for their health (1).

Anabolic steroids are androgens that possess virilizing (androgenic) actions and, consequently, serve to stimulate differentiation and maintenance of the androgen-dependant tissues of the male reproductive system. These hormones also play an important role in facilitating protein synthesis (anabolic actions) in androgen-sensitive tissues such as skeletal muscle, kidney, and bone (25).

A) Structure and Binding

Anabolic steroids are more precisely characterized as anabolic-androgenic steroids. They include testosterone, the primary male sex hormone, and all the chemically altered derivatives of testosterone that have anabolic and androgenic properties. Synthetic anabolic steroids are reported to have greater anabolic than androgenic activity when compared with testosterone (9). In large doses, however, the synthetic steroids also have strong androgenic affects (9,15).

Testosterone was first isolated in 1935 and synthesized artificially shortly thereafter. It was shown to have an anabolic effect in certain pathologic states such as in males who had been castrated and in burn victims. Rumours suggesting the use of synthetic testosterone by athletes were confirmed in the 1952 Olympic Games in Helsinki

(13,31,32). Dr. John Ziegler, of the United States returned convinced that the East Block countries were using these synthetic drugs and proceeded to develop and introduce Dianabol, which until recently, has been the most popular drug used by individuals taking anabolic steroids (2). In 1968, the IOC banned the use of anabolic steroids by athletes and drug testing became routine at the 1976 Montreal Olympics (2,15).

Naturally occurring androgens contain 19 carbon atoms and do not have a side chain attached to the steroid nucleus like synthetic androgens (12,13). Testosterone is the parent compound for anabolic steroids. (See Figure 1).

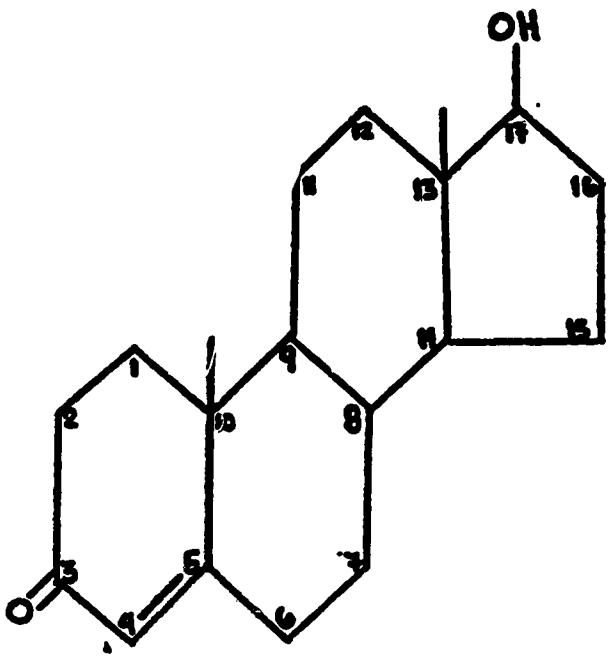


Figure 1: Structure of testosterone hormone. (Adapted from Goldberg 1988)

It is modified in a variety of ways to decrease its androgenic effects, enhance its anabolic effects, increase its bioavailability and duration of action, and minimize its adverse effects (22,36). Alkylation of the 17a position increases anabolic effectiveness after oral administration. (See Figure 2). Esterification of the 17b position prolongs the duration of action after intramuscular administration and produces a compound with fewer hepatic side effects (10,16). (See Figure 3).

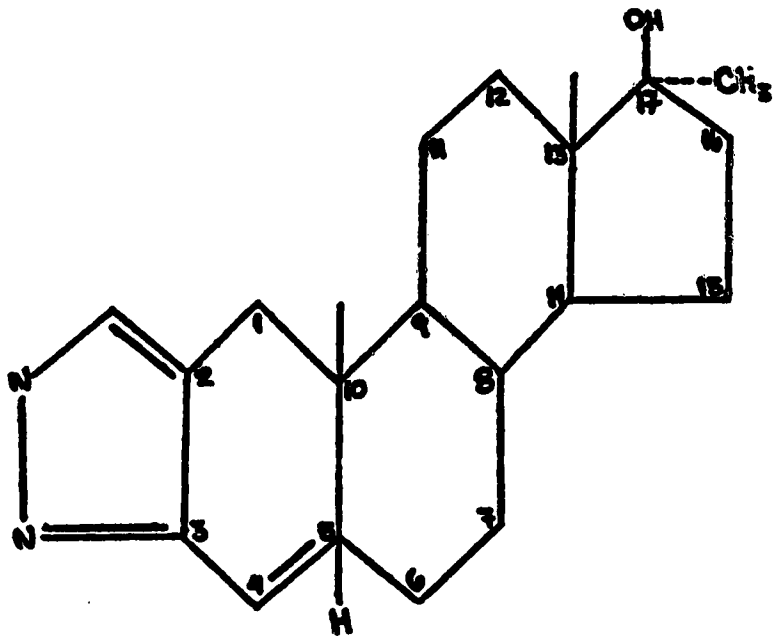


Figure 2: Structure of Stanozolol, 17a-alkylated synthetic anabolic steroid.

(Adapted from Goldberg 1988)

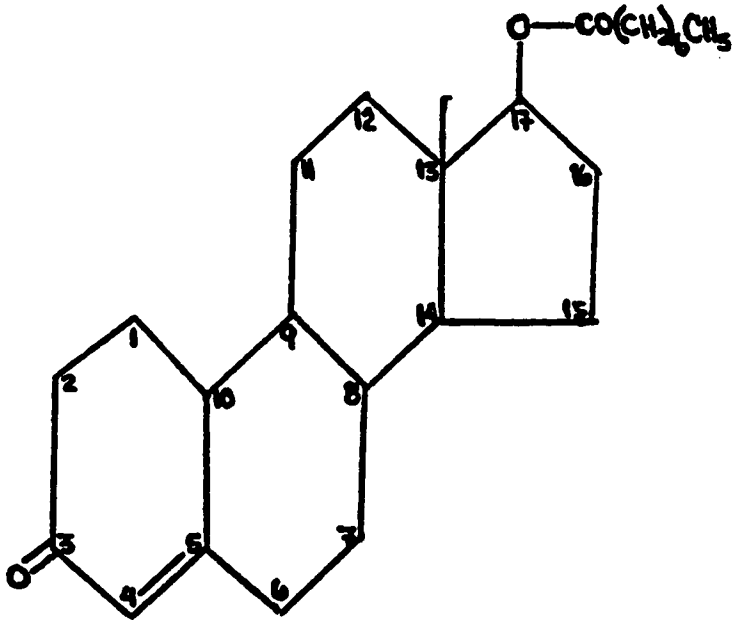


Figure 3: Structure of Nandrolone decanoate (Decadurabolin), 17b-esterified synthetic anabolic steroid. (Adapted from Goldberg 1988)

Steroid hormones in the body are synthesized principally in the testes, adrenal cortex, ovaries and placenta, but the specific secretory products of each of these organs vary considerably (12). The common precursor to all steroid hormones, including androgens, is cholesterol. All steroid secreting cells have the capacity to synthesize cholesterol, but many are also able to sequester circulating cholesterol for androgen production. The relative importance of intracellular cholesterol synthesis versus removal of cholesterol from the blood for use as substrate for hormone production varies from tissue to tissue. Regardless of the its source, most cholesterol is stored in large quantities in steroidogenic tissues in the form of cholesterol esters. When substrate for androgen synthesis is needed, the esters are enzymatically hydrolysed to generate free cholesterol (23).

A large fraction of each steroid hormone in the blood is reversibly bound to

A large fraction of each steroid hormone in the blood is reversibly bound to plasma protein. It is important to appreciate that it is only the unbound or free fraction of hormone that is biologically active; thus no physiological effects are exerted by most of the circulating androgen pool. Protein-bound testosterone comprises approximately 97 to 99% of the total amount in the blood. Only the remaining 1 to 3% is able to interact with receptors in target tissues and initiate biological responses (12). On the other hand, the protein-bound hormone is in reversible equilibrium with the free fraction and provides for a large hormone reserve to replenish the free pool (12). In addition, protein binding protects the hormone from enzymatic degradation by the liver, thereby extending the duration of action or biological half-life of the hormone (13,23).

In contrast to protein hormones, there is no known mechanism for the storage of steroid hormones after they are synthesized. Since steroids are highly lipophilic molecules, they simply diffuse through the vasculature once they are produced. Thus, synthesis and secretion of a steroid hormone tend to be tightly coupled processes. The secretion of androgens is regulated by modulation of the rate of synthesis (12). Testicular androgen synthesis is controlled by negative feedback. This control mechanism involves the hypothalamus and the anterior pituitary gland. Gonadotropin-releasing hormone from the hypothalamus enters the hypophyseal portal vascular plexus and causes the anterior pituitary gland to release luteinizing hormone, which affects the testes and the Leydig cells which produce testosterone (12). Biofeedback occurs when the produced testosterone circulates through the vasculature and makes its way to the hypothalamus which inhibits the release of gonadotropin releasing hormone (12).

Steroid hormone actions generally involve their binding to proteins, called receptors, in the cytoplasm of target cells (11). These steroid receptor-complexes must then undergo a two-step process prior to binding to the nucleus and the regulation of expression of specific genes (11,18). The first step, called activation, involves a conformational change that results in an increased affinity of the steroid receptor complex for the nucleus and polyanions (12,13). The second step, called translocation, involves movement of the activated complex to the nucleus and subsequent binding to chromatin acceptor sites (12,13). Considerable evidence has accumulated to indicate that the availability of receptor sites for binding is a rate-limiting step for steroid action in most mammalian target organs. Tissues without receptors have been shown to be insensitive to steroid action (17).

Separate receptors for androgens, glucocorticoids and estrogens have been demonstrated in skeletal muscle (5,6,24,33). However, there does not appear to be a unique anabolic receptor in muscle, because it has been shown that a number of synthetic anabolic steroids, as well as the natural androgens, bind with the androgen receptor (29).

The existence of steroid receptors in skeletal muscle provides evidence that muscle can be the target of anabolic steroid action. However, the number of steroid receptors may limit the extent of the biological response of muscle (9,10,12,13,15). The existence of specific anabolic receptors for skeletal muscle has been argued, but the data strongly supports the opposite indicating the skeletal muscle contains androgen receptors very similar or identical to that in secondary sex organs (29).

Although anabolic steroids can increase both the size and strength of muscle, this

effect is maintained only as long as the use of anabolic steroid is continued (9). Following discontinuance of the steroid, a significant percentage of the increased size and strength disappears (9). This loss seems to be related to the seriously depressed levels of natural testosterone in the athlete who abuses anabolic steroids (9). The loss is not related to water retention since tissue water content before and after steroid use is not changed (21).

In muscle, increased amounts of actin and myosin are produced under the influence of anabolic steroids (15). These force-producing contractile proteins result in increased muscle strength. Anabolic steroids have more dramatic effects on nitrogen retention and muscular development in females, in boys and in men who have been castrated or are otherwise deficient in natural androgens (15). Anabolic steroids may also exert positive effects on the growth of skeletal muscle by inhibiting protein breakdown (14,26). Takala suggests that the combination of exercise and anabolic steroid causes an increase in collagen concentration in striated muscle (34). To achieve a positive therapeutic result with anabolic steroids, an athlete must have been previously trained in intense weight training and maintain a diet adequate in carbohydrates and proteins (9,11,16). It has been suggested by other investigators that elite body builders do not only increase muscle mass by hypertrophy, but that hyperplasia does seem to occur as well (21,25). This may explain why body builders can maintain a great deal of the gains in muscle mass after interruption of steroid use for at least 12 weeks (21). Soborido states that slow-twitch muscle was significantly enhanced by the administration of anabolizing agents more so that fast-twitch; thus depicting the effect of the hormone

on the sacrotubules of the mitochondria (30). Another interesting finding regarding exercised muscle is that following 30 minutes of recovery, a group without anabolic steroid treatment has significantly higher lactate concentrations than a group with anabolic steroid treatment.

For the elimination of steroids in the body, most steroid hormones are structurally modified by a variety of enzymatic processes prior to their elimination. Although such modification may occur in various tissues, the major site of steroid metabolism is in the liver (9,12). Metabolism usually results in the formation of products that are less active than the parent compound, but in some instances the reverse may occur (9). Thus, the rate of hepatic metabolism is usually closely correlated with the rate of steroid elimination (9,12).

B) Effects

The myotropic activity of androgens has been known for many years (27). Striated muscles are more developed in male than in female mammals (20), and growth of a number of skeletal muscles is increased by androgens in different species (20,27). However, it has not been definitively established whether the myotropic effect of testosterone is accounted by a direct action of the hormone on the muscle, presumably by interaction with an intracellular receptor as in other steroid hormone target organs, or whether the androgen is active through some indirect mechanism, for example an effect on extramuscular production of a myotropic hormone (24).

Anabolic steroids can affect the inflammatory response in soft tissues. They also

affect the immune system, cause fibrolytic changes, and alter glucose homeostasis (10,25).

Anabolic steroids are used in clinical medicine to treat the following states:

Testosterone deficiency

Catabolic or protein-depleting states

Anemia

Carcinoma of the breast

Hereditary angioedema

Osteoporosis

Arthritis

Bone Mass may also be increased but only when the individual exercises also (7).

Decadurabolin (Nandrolone Decanoate injection) is an anabolic steroid that is often used in the treatment of conditions in which a tissue-building action is desired, such as in retarded growth and certain endocrine deficiencies, disease accompanied by protein wastage, negative nitrogen balance or failure to build body protein (4).

There are some contraindications to the use of anabolic steroids that should be noted including liver disease, cardiac-renal failure, nephrosis, pregnancy, carcinoma in some females, and hypersensitivity to specific anabolic steroids (4,10). In fact, besides the known virilizing and hepatotoxic effects, anabolic steroids also induce an atherogenic lipoprotein pattern by decreasing the high-density lipoprotein (HDL) levels and increasing the low-density lipoprotein (LDL) cholesterol levels (1). It becomes important to know

how quickly these effects develop and how quickly and completely they resolve after drug cessation. In a study by Cheung of hyperlipidemic subjects, a reduction of 45% in HDL level was reversed 1 month after drug cessation (3).

Adverse effects are numerous, and range from hepatic, endocrine, cardiovascular and skeletal damage to subjective effects such as aggressiveness, change in libido, muscle spasm, nervous tension, nausea and euphoria (2,4,8,9,15,16,26,36). An equally disturbing and potentially more devastating effect of anabolic steroids is psychological addiction. Athletes who take anabolic steroids usually do so out of insecurity (35).

C) Summary

Anabolic steroids have both anabolic and anticatabolic effects. The anabolic effects result from their ability to attach to the androgenic muscle receptors, their ability to induce a positive nitrogen balance, cause an associated increase in androgenous growth hormone release, and produce motivational and placebo effects. The anticatabolic effects also include reversal of the catabolic effects of increased androgenous cortisol produced during intense training. Anabolic steroids may also reverse an inflammation-induced catabolic state that results from increased cortisol release (10,18,23,26).

ELECTRICAL STIMULATION

A resurgence of interest in electrical stimulation to augment or maintain muscle strength by rehabilitative personnel occurred during the 1980's (10,19,20,25,32). While the initial enthusiasm has somewhat decreased, it is still a frequently used modality. Despite therapists' current ability to elicit intense, comfortable muscular contractions in patients with reduced or absent voluntary control, fundamental questions remain regarding the manner in which electrical stimulation activates human skeletal muscle. It has been demonstrated under a variety of conditions that skeletal muscle adapts to the level of use imposed upon it (34,37).

When a muscle contracts as a result of electrical stimulation, the changes taking place within the muscle are similar to those associated with voluntary contraction. There is a rise in metabolism, with a consequent increase in the demand for oxygen and foodstuffs, and a rise in output of waste products, including metabolites. The hypothalamus activated by the metabolites causes dilation of the capillaries and arterioles, and there is a considerable increase of the blood supply to the muscle. As the muscle contracts and relaxes, there is a pumping effect on the lymphatic vessels and thus an increase in lymphatic return (15).

Studies suggest that functional electrical stimulation might decrease or delay atrophy (13,15,16). Other literature reports that it may help regain muscle function lost after lower motor neuron lesions have been sustained (15,28,29). It has been reported that electrical stimulation reduces post-operative rehabilitation time (5,13,15), aids in the

correction of flexion contractures (3,15) and improves or augments muscle function (3,11,15).

It is clear that chronic muscle stimulation using implanted electrodes alters skeletal muscle properties in a well-defined manner. It is still not clear whether this modality applied clinically actually strengthens muscle or otherwise improves its conditions. During normal cage activity in small animals, a progression of changes are observed in which the muscle first changes its metabolic and then its contractile properties to become a slow-twitch muscle (37). These changes have been documented in rabbit tibialis anterior (21) and rat extensor digitorum longus muscles (8). The fast-to-slow twitch transformation that occurs, is detectable by measurement of muscle contractile, ultrastructural (23), histochemical (8,21), biochemical (8) and/or morphological (8,21) properties. The earliest observable changes occur within a few hours after the onset of stimulation when the sarcoplasmic reticulum begins to swell. Within the next 12 days, there is increased metabolic activity in the muscle (12,21). With prolonged stimulation (28 days), all fibres converted to the oxidative types (21).

A) Currents

The use of electrical currents to promote healing in injuries to the musculoskeletal system is not a novel idea. Both Stevens (41) and Mott (30) reported successfully treating an ununited fracture of the tibia with electrical stimulation in 1816 and 1820. These pioneers concluded that the vital link between form and function in bone is electricity and that mechanical stresses lead to the generation of piezoelectricity which

in turn leads to the formation of callus (30,40,41). The piezoelectric effect is associated to the main organic constituent of the tissue. In the case of bone the main constituent is collagen. The actual effect is a displacement of charge due to the distortion of cross-linkages in the molecular structure. In collagen it would be a disruption of the hydrogen bonds.

There is little evidence to support the belief that electrical muscle stimulation enhances a strengthening program for normal muscle. Interest in this area arose in the late 1970's when Kots claimed a technique of electrical muscle stimulation which utilises a medium frequency current with a 10 second contraction followed by a 50 second rest repeated 10 times per session, could significantly increase muscle strength in athletes. His results remain unsubstantiated (24).

The major criteria for selection for the electrical stimulation treatment is muscle weakness; the aim of treatment is to facilitate strengthening of muscle. Acute conditions should be expected to respond more favourably initially due in part to the short length of time of muscle disuse (6,31). Any condition that results in muscle disuse or atrophy is suitable for strengthening using electrical stimulation (24).

There are no definitive treatment techniques for electrical stimulation, however, many guidelines are necessary in developing treatment protocols. There are many different types of machines and currents used in treatment, including interferential, high voltage, galvanic, and high frequency, however, evidence suggests that low frequency currents are one of the most appropriate in facilitating muscle strength gains (31). A frequency range of 20 to 50 pulses per second provides a high enough rate to produce

muscle tetany, yet not so high as to cause fatigue (31). After the above parameters are appropriately set, the therapist must consider duty cycle (current on and off times). The duty cycle initially is a 1:2 or 1:3 ratio. In the very early stages of rehabilitation, a larger ratio may be necessary to reduce fatigue, especially if the muscle is unable to move the joint throughout its range (31). As rehabilitation progresses, so does the duty cycle ratio, and though a 1:1 ratio may be used, earlier fatigue may occur as a result (31).

B) Effects

As electrical current travels through a conductive biologic medium, alteration in physiologic processes occur at various levels in the total system. At each level, many different processes can be affected, and the modulation used enhances or suppresses the respective physiologic activities. Furthermore, the effect of the stimulation is direct, indirect, or both. At the cellular level, the effect is always direct and deals mostly with the excitation of peripheral nerves, changes in membrane permeability of non-excitatory cells, and modification of fibroblasts and fibroclastic formation (20). At the tissue level, electrical stimulation causes skeletal muscle contraction affecting muscle strength, contraction speed, reaction time and fatiguability, and induces changes in tissue thermal and chemical balance (2,21,31). At the segmental level, the major effect of electrical stimulation is on muscle pumping of lymphatic, venous and arterial flow (31). The final level that electrical stimulation affects, is the systemic level, where it produces an analgesic effect associated with endogenous polypeptides, such as beta-endorphins,

enkaphalins, dopamines and dymophins (15,31).

The physiological responses of non-excitatory cells to electrical stimulation depend in part, on the magnitudes of internally generated electrical potentials. The source of these potentials seems to be associated with the concentration gradient of ions across the cell membrane (31,35). Changes in the concentration gradient may be linked with some ion species, but clinical evidence that the repair of dermal and subdermal connective tissues can be accelerated by externally applied electric current is inconclusive (14,31).

During increasing voluntary effort in humans, normal motor unit recruitment follows the principle that the size of the motor-neuron cell body determines the order in which motor units are activated. Initially, slow-twitch motor units (type I) are recruited followed by the fast twitch motor-units (type II) (18,39). Studies involving electrical stimulation have suggested selective and preferential recruitment of fast-twitch motor units (39).

Most electrical stimulation studies deal with two types of trauma:

1) Models in which injury is produced, by cutting, crushing, and the like, but the tissues are left in their original position or are reapproximated.

2) Models in which a portion of tissue is excised and the remaining tissues are not reapproximated (6).

Studies show that several cell types that normally migrate or grow long distances in embryos respond directionally to surprisingly small fields of current. Also, developing embryos produce substantial endogenous currents. These two facts raise the possibility

that endogenous electrical fields are involved in long-range signalling during embryonic development and during certain cellular responses to injury (36).

Studies have shown that collagen fibres or fibroblasts are affected by pulsed electricity (1,4,40). There are also reports of "better" collagen organization within early callus, hyaline cartilage production in the vicinity of healing osteotomies, and a more rapid return of medullary circulation (6,9). Reversal of the polarity of the field does not affect these results (4). Most alterations occurring between the different injured tissues are dependant upon the field strength of the electrical stimulation (4,33).

It has been reported that during the formation of bone around an electrically stimulated intramedullary stainless steel wire, the impedance between anode and cathode increased by a third (10). Although this rise could be related to bone formation, the experimental design did not allow precise localization of this change (10,33). There was no tendency for bone to form directly around the cathode wire (10).

In a study by Bourguignon and Bourguinon (7), the effect of the position of the cells relative to the electrodes in relation to protein and DNA synthesis by human fibroblasts growing in vitro, was examined. It was shown that DNA synthesis and fibroblast were stimulated to proliferate (4,6,7).

Soft-tissue healing (i.e. wound healing) is defined in terms of the wound module. This complex of tissues and sequential cell populations moves into a wound from its edges during healing by secondary intention. In successive stages following injury, acute inflammation, tissue repair, and remodelling are observed. The acute phase response may last for up to 72 hours and constitutes a non-specific reaction involving both cellular

and humoral elements (22). The humoral response involves blood-borne factors which are released in response to trauma. The cellular response involves the degranulation of mast cells and the release of histamines and prostaglandins (22). The repair phase may last from 48 hours to 6 weeks and is characterized by the synthesis and deposition of collagen. The remodelling phase may last 3 weeks to 12 months or more, and is a period in which collagen is remodelled to increase the functional capabilities of the tendon or ligament or muscle in order to withstand the stresses imposed on it (22). Unfortunately, most soft-tissue healing studies are only evaluated at relatively late post-injury times, thus obscuring the origins of the observed effects (1,6,40).

Studies in animals have established that various forms of electrical stimulation positively affect the course of soft tissue growth, repair and remodelling (6). Electrical stimulation enhances the repair process of ligaments by causing a change in the ratio of collagen types from immature type III to normal type I collagen (1,22).

Electrical stimulation has been used for many years on skeletal muscle but because its a physiological basis has yet to be understood and there is no general agreement on stimulation parameters, doses, or conditions (26).

There are three major differences between most animal and human studies

-1- Chronic animal electrical stimulation is usually accomplished using implanted electrodes, while human muscle is usually stimulated transcutaneously (28).

-2- The stimulation doses used in animal studies have been 10 to 100 times greater than those used in human studies.

-3- Human muscle has been stimulated isometrically, while animal limbs were allowed to move freely (26).

C) Summary

Studies on soft tissue stimulation are not sufficiently advanced and do not provide information concerning the likely success or failure of any given stimulation modality. More animal studies on the effects of electrical stimulation on tissue growth, repair and remodelling are clearly required.

Chapter 3

Methods and Procedures

ANIMALS

All procedures described below were carried out in conformance with the guiding principles for the care and use of animals of the Canadian Council on Animal Care and the American Physiological Society. Thirty-seven male Sprague-Dawley rats, weighing 180-200g at the start of the experiment, were housed in cages of two or threes and provided with commercial rat chow ("Wayne Lab Blox") and water ad libitum. The animals were kept on a 12h light - 12h dark cycle in an environmentally stable temperature of 20 C.

EXERCISE TRAINING PROGRAM

The familiarisation phase lasted 3 to 5 days and consisted of a twice a day 10 minute run at 10m/min, 0% grade.

After the initial familiarization to running, the rats were progressively overloaded by a gradual increase in speed, duration and grade, until 60 minutes of continuous endurance exercise was achieved (2,7,8). Appendix 4 outlines the proposed training

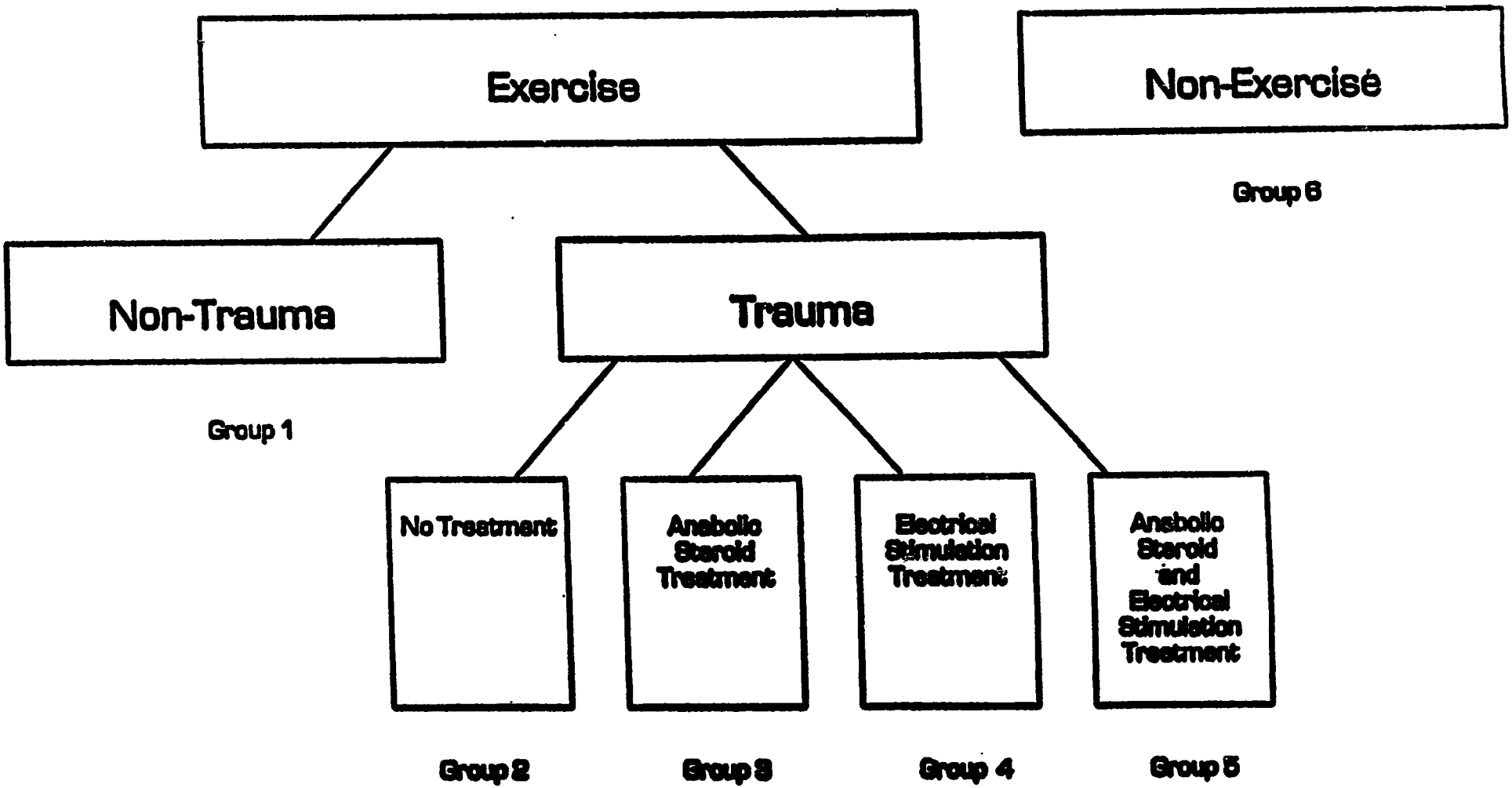
regime. This regime was used as a guideline and only slight modifications occurred to accommodate individual adaptations to the program.

Each training session was preceded by a warm-up session which lasted 4-5 minutes and included a progressive speed increase to reach the desired speed for that day. Each session also included a cool down of 2-3 minutes (8).

TISSUE TRAUMA

The thirty-seven animals were weighed and these measurements recorded on the trauma day. The trauma day occurred once the exercise protocol was entirely completed. The animals were randomly assigned by weight to one of the experimental groups. Experimental group 1 (See Figure 4), received no trauma. Experimental groups 2-3-4-5 were subjected to a single impact trauma to the medial aspect of the calf of the right leg using a blunt injury device which is the adaptation by Fisher (3) of the method of Stratton (6). During the procedure, the rats including controls were briefly anaesthetized with halothane. The trauma was delivered by dropping a solid aluminum bar with a flat impact surface only once, down a tubular guide through a distance of 125mm onto the muscle (1,38cm diameter, 700g). The force delivered by the device was 0,57 Newton/meter/cm2, and the moment of impact was 1099 kg meter/sec. The rat limb was positioned manually so the device was directed over the muscle belly of the calf to avoid

Figure 4: **Effects of anabolic steroids and electrical stimulation on collagen production in rat traumatized muscle**



any fracture of the tibia. This procedure has been described in detail elsewhere (3). The day of trauma was considered Day 1 of the experiment.

TISSUE TREATMENT (See Figure 4)

A) Groups 1-2 received no treatment for 10 days following the trauma.

B) Group 3 was treated with anabolic steroid intramuscular injections in the muscle belly of the right calf. This injection consisted of 5mg of Decadurabolin on Day 1 of the experiment. (See Appendix 1)

C) Group 4 was treated daily with transcutaneous electrical stimulation on the right gastrocnemius using a Focus NMS unit. (ANSI/AAMI NS4-1985). (See Appendix 2 and 5)

D) Group 5 was treated with both anabolic steroids and electrical stimulation with the same dosages and treatment protocol as groups 3 and 4. (See Appendix 1, 2 and 5.)

E) Group 6 was not exercised and not traumatized.

TISSUE SAMPLING

The thirty-seven animals were weighed on the tissue sampling (Day 10) and all the muscles that were excised from each animal were weighed individually. These measurements were recorded. The animals were sacrificed at the end of day 10 by anaesthesia and subsequent cervical dislocation. The right and left medial gastrocnemius and the soleus were excised from the hind limb of the animals of Group 2-3-4-5, weighed and frozen in liquid nitrogen. For group 1, only the right medial gastrocnemius and soleus were removed, weighed and frozen in liquid nitrogen. The muscles were then put in individually identified vials and stored (-70C) for subsequent analysis using the method of Woessner (1) for collagen content and Lowry's non-collagenous protein determination (4). (See Appendix 3).

The left lateral gastrocnemius was removed in 2 rats from Group 1 and 1 rat of Groups 2-3-4-5, weighed and frozen in liquid nitrogen. The muscles were then put in individually identified vials and stored (-70 C) for subsequent analysis using the citrate synthase assay for training effect. Group 6 was sacrificed and the right and left lateral gastrocnemius were excised according to the above mentioned method. The untrained muscles harvested were also analyzed with the citrate synthase assay (5). (See Appendix 3).

STATISTICAL ANALYSIS

The citrate synthase data was analyzed using a student t-test comparing the trained and untrained group and a significant difference at the 95% confidence interval ($p < 0.05$) was accepted.

The non-collagenous protein content was analyzed for each muscle, soleus or medial gastrocnemius, and for each limb, the traumatized or the contralateral limb, using a one-way analysis of variance (ANOVA). A posteriori comparisons were made with the student Newman-Keuls method and significant differences at the 95% confidence interval ($p < 0.05$) or better were accepted.

The same statistical tests, as used for the non-collagenous protein, were used for the analysis of the collagen content.

Chapter 4

Results

In order to assess the effects of trauma, anabolic steroids and electrical stimulation on non-collagenous protein and collagen content, the intact medial gastrocnemius and soleus were dissected from healthy controls as well as from both legs of the injured rats. All rats were exercised except the control group which was used to evaluate the citrate synthase content. This last statement implies that there was an exercised control group which did not receive any trauma.

TRAINING EFFECT

A significant increase ($p<0.05$) in the amount of citrate synthase in the lateral gastrocnemius muscle was found in the trained rat compared to the untrained rat following a seven week exercise program. (Table 1).

TISSUE TREATMENT

There was no significant difference found in non-collagenous protein content between the injured rat and the injured rat treated with anabolic steroids at the site of injury (Table 2).

Anabolic steroids created a significant increase ($p<0.05$) in the amount of collagen in the medial gastrocnemius of the injured limb compared to the untreated rat.

Table 1. EFFECTS OF TRAINING ON CITRATE SYNTHASE

	Lateral Gastrocnemius (umoles/ min per g)
Control rat	28.2[a]
Trained rat	37.7[a]

Values with the same alphabetical suprascript are significantly different from each other ($p<0.05$) ($n=6$ control, $n=7$ trained rat)

The changes also showed a significant decrease ($p<0.05$) in collagen content of the soleus muscle of the injured limb compared to the contralateral limb of the same subjects (Table 3).

Electrical stimulation was found to decrease the amount of non-collagenous protein in the medial gastrocnemius muscle of the injured limb. A significant decrease ($p<0.05$) was seen in the medial gastrocnemius of the uninjured limb treated with electrical stimulation compared to the medial gastrocnemius in the uninjured limb of the untreated rat. The changes showed significance ($p<0.05$) in the amount of non-collagenous protein in the soleus muscle in the injured limb of the injured rat treated with electrical stimulation compared to the same subjects soleus muscle contralateral limb (Table 4).

Electrical stimulation in the traumatized limb was found to decrease collagen content in the medial gastrocnemius but increase collagen content in the soleus when compared to the injured untreated rat. A significant decrease ($p<0.05$) in collagen content was found comparing the medial gastrocnemius muscle of the uninjured untreated limb of the injured rat with the medial gastrocnemius muscle of the uninjured limb of the rat treated with electrical stimulation. The changes also showed a significant decrease ($p<0.05$) in the amount of collagen in the soleus muscle of the injured limb of the untreated subjects compared to the contralateral soleus muscle of the same group (Table 5).

The soleus muscle treated with anabolic steroids and electrical stimulation was found to be significantly lower ($p<0.05$) in non-collagenous protein content in the

Table 2. EFFECTS OF TRAUMA AND ANABOLIC STEROIDS ON NON-COLLAGENOUS PROTEIN CONTENT 10 DAYS POST-INJURY.

	Medial Gastrocnemius	Soleus
	(ug of non-collagenous protein/ mg of muscle)	
Control	319.0	266.4
Injured rat		
Injured limb	347.4	331.1
Uninjured limb	338.0	298.6
Injured rat treated with anabolic steroids at the injury site		
Injured limb	283.0	299.5
Uninjured limb	317.7	318.5

No significant differences were observed between treatments for any of the muscles studied (n=6 control, n=7 injured rat).

Table 3. EFFECTS OF TRAUMA AND ANABOLIC STEROIDS ON COLLAGEN CONTENT 10 DAYS POST-INJURY.

	Medial Gastrocnemius	Soleus
	(ug of collagen/ mg of muscle)	
Control	3.06	5.40
Injured rat		
Injured limb	3.14[a]	4.52[b]
Uninjured limb	4.75	8.24[b]
Injured rat treated with anabolic steroids at the injury site		
Injured limb	5.37[a]	6.41
Uninjured limb	3.96	5.40

Values with the same alphabetical superscript are significantly different from each other ($p<0.05$) (n=6 control, n=7 injured rat).

Table 4. EFFECTS OF TRAUMA AND ELECTRICAL STIMULATION ON NON-COLLAGENOUS PROTEIN CONTENT 10 DAYS POST-INJURY.

	Medial Gastrocnemius	Soleus
	(ug of non-collagenous protein/ mg of muscle)	
Control	319.0	266.4
Injured rat		
Injured limb	347.4	331.1
Uninjured limb	338.0[b]	298.6
Injured rat treated with electrical stimulation at the injury site		
Injured limb	319.1	333.1[a]
Uninjured limb	260.6[b]	255.7[a]

Values with the same alphabetical superscript are significantly different from each other ($p<0.05$) (n=6 control, n=7 injured rat)

Table 5. EFFECTS OF TRAUMA AND ELECTRICAL STIMULATION ON COLLAGEN CONTENT 10 DAYS POST-INJURY.

	Medial Gastrocnemius	Soleus
	(ug of collagen/ mg of muscle)	
Control	3.06	5.40
Injured rat		
Injured limb	3.14	4.52[b]
Uninjured limb	4.75[a]	8.24[b]
Injured rat treated with electrical stimulation at the injury site		
Injured limb	2.67	7.82
Uninjured limb	2.59[a]	8.78

Values with the same alphabetical suprascript are significantly different from each other ($p<0.05$) (n=6 control, n=7 injured rat).

injured limbs than the soleus muscle in the injured limbs which was untreated or treated with electrical stimulation only. The medial gastrocnemius muscle treated with electrical stimulation was significantly lower ($p<0.05$) in non-collagenous protein content in the uninjured limb than the medial gastrocnemius muscle in the uninjured limbs which was untreated or treated with anabolic steroids and electrical stimulation. The soleus muscle for the group treated with anabolic steroids and electrical stimulation had a higher non-collagenous protein content than the other groups: untreated, treated with anabolic steroids only or treated with electrical stimulation only. The group treated with anabolic steroids and electrical stimulation was found to have a significantly higher ($p<0.05$) non-collagenous protein content in the uninjured limb than the same limb of the group treated with electrical stimulation only. The changes also showed a significant increase ($p<0.05$) in the non-collagenous protein content of the soleus muscle of the injured limb of the group treated with anabolic steroids compared to its contralateral soleus. A significant decrease ($p<0.05$) in the non-collagenous protein content of the soleus muscle in the injured limb of the group treated with anabolic steroid and electrical stimulation was also found compared to it's contralateral soleus muscle (Table 6).

A significant increase ($p<0.05$) was seen in the amount of collagen of the medial gastrocnemius muscle of the injured limb treated with anabolic steroids when compared with the medial gastrocnemius muscle of the injured limb of the untreated group and the group treated with electrical stimulation. The collagen content of the medial gastrocnemius of the uninjured limb of the untreated rat or the rat treated with anabolic steroids was found to be significantly higher ($p<0.05$) than that of the medial

Table 6. EFFECTS OF TRAUMA, ANABOLIC STEROIDS AND ELECTRICAL STIMULATION ON NON-COLLAGENOUS PROTEIN CONTENT 10 DAYS POST-INJURY.

	Medial Gastrocnemius	Soleus
	(ug of non-collagenous protein/ mg of muscle)	
Injured rat		
Injured limb	347.4	331.1[a]
Uninjured limb	338.0[c]	298.6
Injured rat treated with anabolic steroids		
Injured limb	283.0	299.5
Uninjured limb	317.7[d]	318.5
Injured rat treated with electrical stimulation		
Injured limb	319.1	333.1[b,f]
Uninjured limb	260.6[c,d]	255.7[e,f]
Injured rat treated with anabolic steroids and electrical stimulation		
Injured limb	305.8	253.2[a,b,g]
Uninjured limb	285.6	341.6[e,g]

Values with the same alphabetical suprascript are significantly different from each other($p < 0.05$)($n=7$)

gastrocnemius of the uninjured limb of the group treated with electrical stimulation or even the group treated with anabolic steroids and electrical stimulation. Changes also showed a significant decrease ($p<0.05$) in the amount of collagen in the soleus muscle of the injured limb of the untreated subjects compared to the soleus muscle of the contralateral limb of the same group (Table 7).

No significance was found in the non-collagenous protein content of both the medial gastrocnemius and soleus muscles in both limbs of the injured rat compared to the control rat (Table 8).

An increase was seen in the collagen content of both the medial gastrocnemius and soleus muscles in both limbs of the injured rat compared to the control rat. Changes also showed a significant decrease ($p<0.05$) in the amount of collagen in the soleus muscle of the injured limb of the untreated subjects compared to the soleus of the contralateral limb of the same groups (Table 9).

For the soleus muscle an increase in non-collagenous protein content was found in the injured limb of the group treated with electrical stimulation when compared to all the other groups. This increase was significant ($p<0.05$) when comparing it to the soleus muscle of the injured limb of the group treated with anabolic steroids and electrical stimulation. The opposite was found in the soleus muscle of the uninjured limb where the group treated with anabolic steroids and electrical stimulation was significantly higher ($p<0.05$) than the uninjured limb of the group treated with electrical stimulation only. A decrease in non-collagenous protein content in the medial gastrocnemius muscle of the uninjured limb of the group treated with electrical stimulation was found to be

Table 7. EFFECTS OF TRAUMA, ANABOLIC STEROIDS AND ELECTRICAL STIMULATION ON COLLAGEN CONTENT 10 DAYS POST-INJURY.

	Medial Gastrocnemius	Soleus
	(ug of collagen/ mg of muscle)	
Injured rat		
Injured limb	3.14[a]	4.52[g]
Uninjured limb	4.75[c,d]	8.24[g]
Injured rat treated with anabolic steroids		
Injured limb	5.37[a,b]	6.41
Uninjured limb	3.96[e,f]	5.40
Injured rat treated with electrical stimulation		
Injured limb	2.67[b]	7.82
Uninjured limb	2.59[c,e]	8.78
Injured rat treated with anabolic steroids and electrical stimulation		
Injured limb	2.63	4.77
Uninjured limb	2.24[d,f]	6.16

Values with the same alphabetical suprascript are significantly different from each other($p < 0.05$)($n = 7$)

Table 8. EFFECTS OF TRAUMA ON NON-COLLAGENOUS PROTEIN CONTENT 10 DAYS POST-INJURY.

	Medial Gastrocnemius	Soleus
	(ug of non-collagenous protein/ mg of muscle)	
Control rat	319.0	266.4
Injured rat		
Injured limb	347.4	331.1
Uninjured limb	338.0	298.6

No significant difference were observed between treatments for any of the muscles studied (n=6 control, n=7 injured rat).

Table 9. EFFECTS OF TRAUMA ON COLLAGEN CONTENT 10 DAYS POST-INJURY.

	Medial Gastrocnemius	Soleus
	(ug of collagen/ mg of muscle)	
Control rat	3.06	5.40
Injured rat		
Injured limb	3.14	4.52[a]
Uninjured limb	4.75	8.24[a]

Values with the same alphabetical suprascript are significantly different from each other ($p<0.05$) (n=6 control, n=7 injured rat)

significant ($p < 0.05$) when comparing it to the medial gastrocnemius muscle of the group that was treated with anabolic steroids only. There were changes which showed a significant increase ($p < 0.05$) in the non-collagenous protein content of the soleus muscle of the injured limb of the group treated with electrical stimulation compared to the contralateral soleus muscle of the same group. A significant decrease ($p < 0.05$) in the non-collagenous protein content of the soleus muscle of the injured limb of the group treated with anabolic steroids and electrical stimulation was also found when comparing to the contralateral soleus of the same group (Table 10).

Collagen content was found to be significantly higher ($p < 0.05$) in the medial gastrocnemius muscle of the injured limb of the group treated with anabolic steroids only compared to the same muscle and the same limb of the control group and the group treated with electrical stimulation only. The medial gastrocnemius muscle of the uninjured limb of the group treated with anabolic steroids was found to also increase significantly ($p < 0.05$) compared to the medial gastrocnemius muscle of the uninjured limb of the group treated with electrical stimulation only and the group treated with anabolic steroids and electrical stimulation (Table 11).

Table 10. EFFECTS OF ANABOLIC STEROIDS AND ELECTRICAL STIMULATION ON NON-COLLAGENOUS PROTEIN CONTENT 10 DAYS POST-INJURY.

	Medial Gastrocnemius	Soleus
	(ug of non-collagenous protein/ mg of muscle)	
Control rat	319.0	266.4
Injured rat treated with anabolic steroids		
Injured limb	283.0	299.5
Uninjured limb	317.7[b]	318.5
Injured rat treated with electrical stimulation		
Injured limb	319.1	333.1[a,d]
Uninjured limb	260.6[b]	255.7[c,d]
Injured rat treated with anabolic steroids and electrical stimulation		
Injured limb	305.8	253.2[a]
Uninjured limb	285.6	341.6[c]

Values with the same alphabetical suprascript are significantly different from each other ($p<0.05$) (n=6 control, n=7 injured rat)

Table 11. EFFECTS OF ANABOLIC STEROIDS AND ELECTRICAL STIMULATION ON COLLAGEN CONTENT 10 DAYS POST-INJURY.

	Medial Gastrocnemius	Soleus
	(ug of collagen/ mg of muscle)	
Control rat	3.06[b]	5.40
Injured rat treated with anabolic steroids		
Injured limb	5.37[a,b]	6.41
Uninjured limb	3.96[c,d]	5.40
Injured rat treated with electrical stimulation		
Injured limb	2.67[a]	7.82
Uninjured limb	2.59[c]	8.78
Injured rat treated with anabolic steroids and electrical stimulation		
Injured limb	2.63	4.77
Uninjured limb	2.24[d]	6.16

Values with the same alphabetical suprascript are significantly different from each other ($p<0.05$) (n=6 control, n=7 injured rat)

SUMMARY

Electrical stimulation was found to increase the non-collagenous protein content in the injured limb. Electrical stimulation was also found to decrease the collagen content in the injured limb. Anabolic steroids were found to increase collagen content in both limbs of the injured rat and was also found to decrease non-collagenous protein in the injured limb. Anabolic steroids and electrical stimulation as a combined treatment were found to decrease both the non-collagen protein content as well as the collagen content of the traumatized muscle (Table 12,13,14,15 and Figure 5,6).

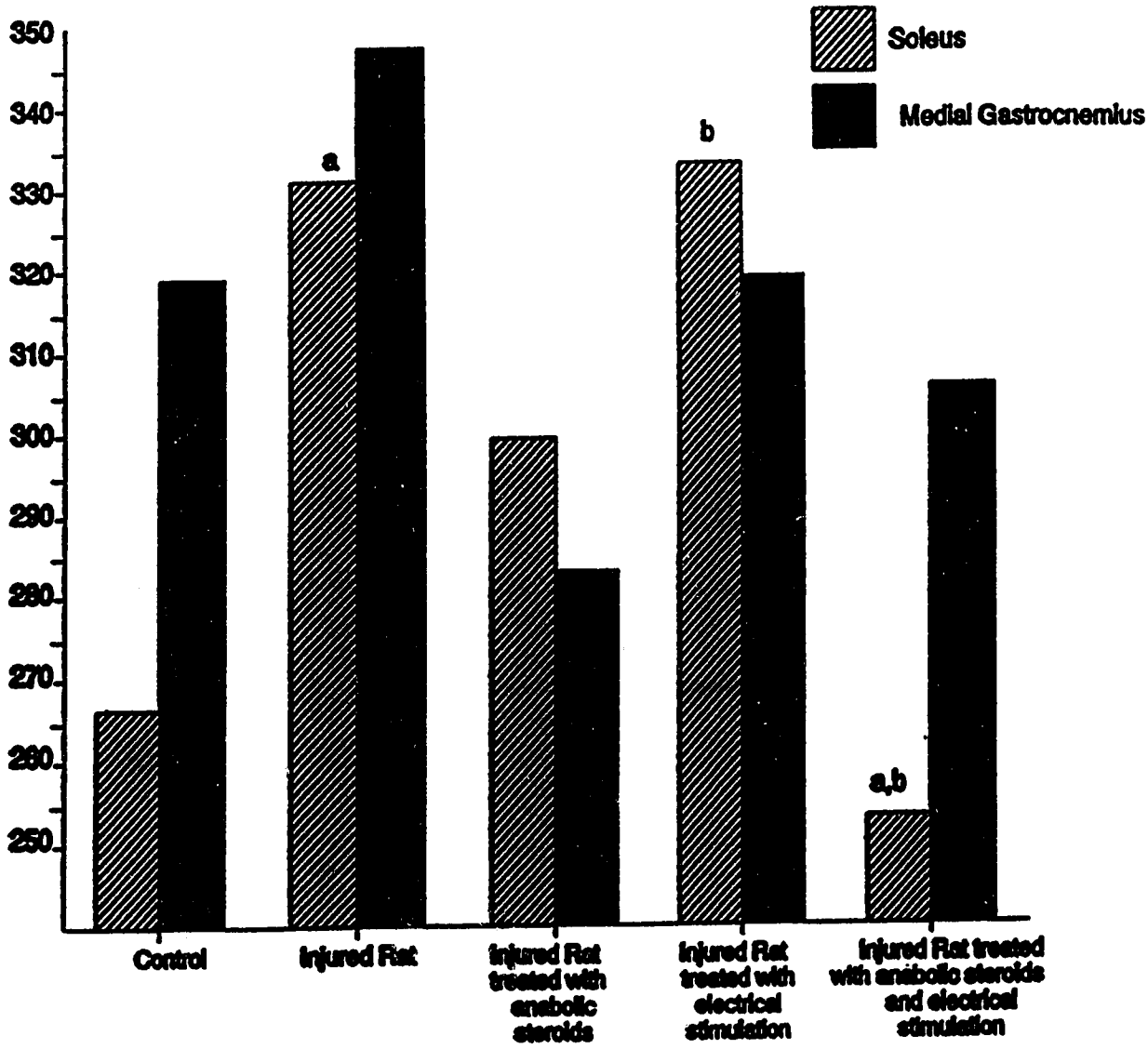
Table 12. MATRIX OF SIGNIFICANT DIFFERENCE IN THE TRAUMATIZED LEG BETWEEN CONTROL, TRAUMA, TREATMENT WITH ANABOLIC STEROIDS, TREATMENT WITH ELECTRICAL STIMULATION AND TREATMENT WITH ANABOLIC STEROIDS AND ELECTRICAL STIMULATION FOR NON-COLLAGENOUS PROTEIN CONTENT.

	Group 1	Group 2	Group 3	Group 4	Group 5
Group 1					
Group 2					A
Group 3					
Group 4					B
Group 5		A		B	

Group 1= Control, Group 2= Trauma, Group 3= Treatment with Anabolic Steroids, Group 4= Treatment with Electrical Stimulation, Group 5= Treatment with Anabolic Steroids and Electrical Stimulation.

The alphabetical codings indicate significant difference between the groups ($p<0.05$) ($n=6$ control, $n=7$ trauma)

Figure 5: **Effects of Trauma, Anabolic Steroids and Electrical Stimulation on Non-collagenous Protein Content 10 Days Post-Injury**



Bars with the same alphabetical coding are significantly different from each other ($p<0.05$)

Table 13. MATRIX OF SIGNIFICANT DIFFERENCE IN THE NON-TRAUMATIZED LEG BETWEEN CONTROL, TRAUMA, ANABOLIC STEROID TREATMENT, ELECTRICAL STIMULATION TREATMENT AND ANABOLIC STEROID AND ELECTRICAL STIMULATION TREATMENT FOR NON-COLLAGENOUS PROTEIN CONTENT.

	Group 1	Group 2	Group 3	Group 4	Group 5
Group 1					
Group 2				A	
Group 3				B	
Group 4		A	B		C
Group 5				C	

Group 1= Control, Group 2= Trauma, Group 3= Treatment with anabolic steroids, Group 4= Treatment with Electrical Stimulation, Group 5= Treatment with Anabolic Steroids and Electrical Stimulation.

The alphabetical codings indicate significant difference between the groups ($p<0.05$) ($n=6$ control, $n=7$ trauma).

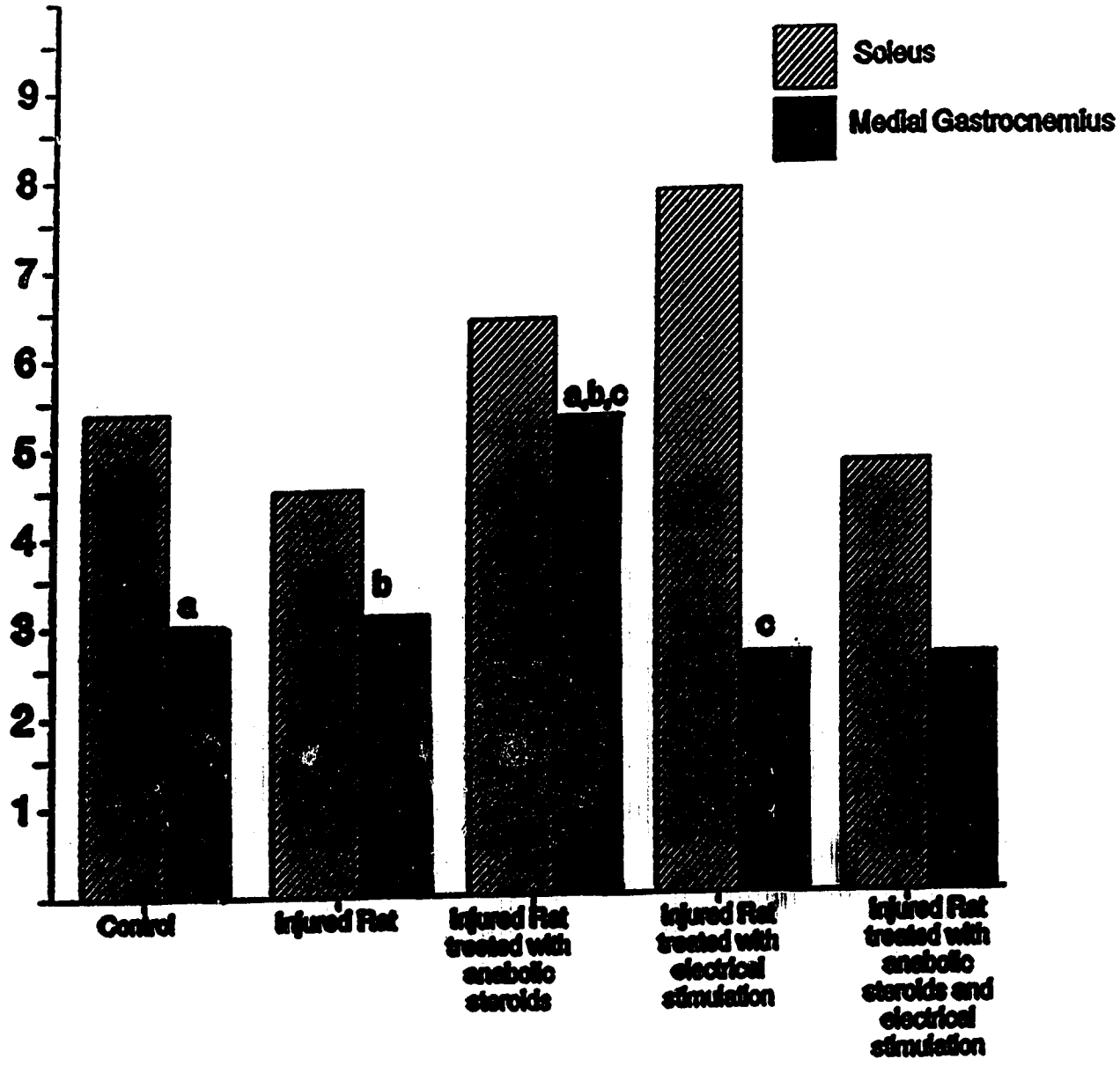
Table 14. MATRIX OF SIGNIFICANT DIFFERENCE IN THE TRAUMATIZED LEG BETWEEN CONTROL, TRAUMA, ANABOLIC STEROID TREATMENT, ELECTRICAL STIMULATION TREATMENT, AND ANABOLIC STEROID AND ELECTRICAL STIMULATION TREATMENT FOR COLLAGEN CONTENT.

	Group 1	Group 2	Group 3	Group 4	Group 5
Group 1			C		
Group 2			A		
Group 3	C	A		B	
Group 4			B		
Group 5					

Group 1= Control, Group 2= Trauma, Group 3= Treatment with Anabolic Steroids, Group 4= Treatment with Electrical Stimulation, Group 5= Treatment with Anabolic Steroids and Electrical Stimulation.

The alphabetical codings indicate significant difference between the groups ($p<0.05$) ($n=6$ control, $n=7$ trauma).

Figure 6: **Effects of Trauma, Anabolic Steroids and Electrical Stimulation on Collagen Content 10 Days Post-Injury**



Bars with the same alphabetical coding are significantly different from each other ($p<0.05$)

Table 15. MATRIX OF SIGNIFICANT DIFFERENCE IN THE NON-TRAUMATIZED LEG BETWEEN CONTROL, TRAUMA, ANABOLIC STEROID TREATMENT, ELECTRICAL STIMULATION TREATMENT AND ANABOLIC STEROID AND ELECTRICAL STIMULATION TREATMENT FOR COLLAGEN CONTENT.

	Group 1	Group 2	Group 3	Group 4	Group 5
Group 1					
Group 2				A	B
Group 3				C	D
Group 4		A	C		
Group 5		B	D		

Group 1= Control, Group 2= Trauma, Group 3= Treatment with Anabolic Steroids, Group 4= Treatment with Electrical Stimulation, Group 5= Treatment with Anabolic Steroids and Electrical Stimulation.

The alphabetical codings indicate significant difference between the groups ($p<0.05$) ($n=6$ control, $n=7$ trauma).

Chapter 5

Discussion

This study undertook to investigate the effects of electrical stimulation and an anabolic steroid (Decadurabolin) on the non-collagenous protein content and collagen content of traumatized skeletal muscle of the hind limb of the rat. The results of this study showed an increase in collagen content of the medial gastrocnemius muscle with the use of anabolic steroids and an increase in non-collagenous contractile protein content in the soleus muscle with the use of electrical stimulation.

TRAINING EFFECT

The rat sample chosen was subjected to a proven training regime (Appendix 4) (12, 41). It is possible to define a training effect because each of the three skeletal muscle fibre types in rodents adapt during exercise training by an increase in their oxidative capacities (4, 21). Baldwin et al. (3) found that under different training conditions, there were significant increases in citrate synthase activity induced in the three types of skeletal muscle fibres. Furthermore, it has been shown that this adaptive response for muscle composed of a mixture of fibre types is increased proportionally to the level of exercise involvement (16). Therefore, the extent of the increase in mitochondrial markers is presumed to reflect the degree of motor unit involvement during exercise (40). Fitts et al. (17) found that the increase in oxidative capacity induced by

exercise training was a linear function of the duration of daily running. Their data included training programs of up to two hours per day which doubled the oxidative capacity in muscles of mixed fibre composition. Terjung (40) trained rats on a rodent treadmill utilizing a program which varied from three times a week once a day, to every day, twice a day. All the programs used by Terjung were found to increase the level of activity of citrate synthase in muscle of mixed fibre composition.

The present findings showed a significant increase in citrate synthase activity in trained rats compared to untrained rats. The values found for the untrained lateral gastrocnemius muscle which is classified as a muscle of mixed fibre composition, were 28.2 umoles/min per g which was significantly less than the 37.7 umoles/min per g for the homologous trained muscle. These values are comparable to those found in a study by Baldwin et al. (4) which stated that untrained muscle of mixed fibre composition yielded 20.6 umoles/min per g and that the homologous trained muscle yielded 37.5 umoles/min per g. The data by Baldwin was also found to be statistically significant and indicative of a training effect.

It is concluded from the above findings that the exercise-trained rats which were used in the present study were properly exercise-conditioned subjects.

TISSUE TREATMENT

The trauma model used was previously established (15,38) and has served as a basis for studies of protein metabolism in trauma. Tischler and Fagan (42) demonstrated alterations in muscle protein, amino acid and carbohydrate metabolism both locally and

systemically using a similar model employing a multiple-blow trauma device.

The healing process produced fibrosis in skeletal muscle by day 3 post-injury and increased by day 6 (15). The present study used day 10 post-injury as the final day of treatment to determine which of the following treatments were the most suited to assist in the restoration of non-collagenous protein versus the formation of collagen: a) anabolic steroid treatment, b) electrical stimulation treatment, or c) combined anabolic steroid and electrical stimulation treatment.

Fibroblasts are specialized cells that develop from embryonic mesenchyme. They play a critical role in morphogenesis, dictating the structure of the skeleton, locations of muscle cells, routes taken by nerve fibres and organization of the skin. In the mature animal, fibroblasts continue to synthesize and maintain elements of the connective tissue matrix. Studies conducted have established that fibroblasts do not function autonomously but, instead, are modulated by distinct molecular signals from T lymphocytes, platelets, neutrophils and macrophages (20,33). Observations have been made regarding the fact that fibroblasts are capable of substantial migration during growth in vitro (55). As fibroblasts migrate, they send out lamellipodia, which adhere to surfaces and allow the body of the fibroblast to be drawn up to the new adhesion site by activation of the contractile filaments within the cell (20). Fibroblasts also have the capability of directed migration towards specific chemoattractants (33). Fibroblasts produce collagen and collagen depositions are part of the scaring process which compromises the subsequent functional capacity of the muscle, because it involves replacement of the contractile tissue with non-contractile, extracelluar fibrillar matrix material.

Skeletal muscle can repair itself independently of other tissues, provided nutrient and oxygen supplies are available. Regeneration of muscle fibres that have been lost due to a variety of traumatic stimuli and the development of new muscle fibres in vitro require the mobilization of a group of proliferating cells, their subsequent fusion, and proliferation (22). There is strong evidence that satellite cells are directly and uniquely involved in this type of proliferation (22). The satellite cells which appear in the damaged area subsequently fuse to form myotubes. These myotubes eventually fuse with the remaining intact ends of the muscle fibres, if present (22). A variety of injuries have elicited a regenerative response with the common characteristic of inducing an activation of the satellite cells so the cells become motile and mitotic (36).

A) Anabolic Steroids

The stimulatory effects of androgens on protein synthesis have been of therapeutic interest (25). The androgens stimulate growth and development of the skeleton and skeletal muscle during puberty (25). The initial use of anabolic steroids in the early 1940's was for the purpose of inhibiting the loss of protein after major surgery, and to stimulate muscle regeneration in debilitating disorders such as muscular dystrophy and diabetes (25).

The present study used a single dose of an anabolic steroid injected into the trauma site and demonstrated that the anabolic steroid promoted the formation of non-collagenous protein above the amount found in the control muscle of the soleus, but was no better than not treating the muscle or treating it with electrical stimulation. It has

been shown by other investigators that androgens act on primary myoblasts to facilitate the establishment of the myogenic cell lines. The foregoing supports the concept that androgens have direct effects on striated muscle cells (28). Beyond these findings it should be noted, that even though all skeletal muscles probably respond somewhat to androgens, there is also considerable variation in the sensitivity of individual muscles to these hormones. Saborido et al. (34) have found that anabolic/androgenic steroids seem to exert their effects preferentially on fast twitch muscles. The two muscles that were used in the present research were the soleus and medial gastrocnemius which are predominantly slow-twitch muscle, and mixed fibre muscle respectively.

The actions of anabolic steroids on muscle are due to anabolic, anticatabolic and motivational effects. The anabolic effect on protein synthesis, is mediated primarily through a shift to positive nitrogen balance as a result of increased nitrogen retention through the greater utilization of ingested protein (26). Therefore, a rational does exist for the use of synthetic hormones in attempts to induce increased muscle mass. This increase in muscle mass continues during anabolic steroid treatment and occurs in both normal and catabolic states (26). If the subjects were in a constant catabolic state, then anabolic steroids would be employed to increase protein synthesis in order to reverse this state and achieve a positive nitrogen balance (26). Anabolic steroids also have an anabolic effect on protein synthesis when they are used in patients to counteract the medically induced catabolic effect of glucocorticoids (26).

The anabolic steroid treatment protocol in the present study did not cause the non-collagenous protein to increase significantly, when compared to the homologous untreated

muscle after trauma. In general, the effect of anabolic steroids is systemic, and therefore extends to the contralateral leg of the rat.

The effect of anabolic steroids on muscle protein turnover is controversial. In other reported animal studies, testosterone propionate has been shown to increase rates of muscle protein synthesis and degradation (27), whereas trenbolone acetate decreases both parameters (35,43). Another anabolic steroid, stanozolol, increased muscle protein synthesis, while protein degradation was not affected (6). Decadurabolin, otherwise known as nandrolone decanoate, was used in the present study. Decadurabolin is one of the synthetic steroid preparations commonly used when a sustained anabolic effect is desired (10). In animal experiments, it has been found that when injected intramuscularly, Decadurabolin is slowly absorbed unchanged from the injection site into the general circulation (7). In rats, the time required for the amount of steroid at the injection depot to decline to 50 percent of the initial amount, was approximately 130 hours (7). This absorption rate would imply that in the present study, in which the muscle was removed after ten days, only a trace of decadurabolin was left, at which time the protein anabolic effect would no longer be taking place.

It has been stated that during development of exercised-induced hypertrophy, the collagen content of skeletal muscle may be increased (39). It was also stated that nandrolone decanoate therapy has a stimulatory effect on collagen production, especially type III collagen (18). In the present study, the collagen content of the medial gastrocnemius muscle and the soleus muscle, in the group treated with anabolic steroids, was found to be significantly higher than in the group which received no trauma, or the

group which received trauma and no treatment. The collagen content was also found to be significantly increased in both the traumatized and in the non-trauma contralateral control leg, compared to the group treated with electrical stimulation. The fact that the contralateral limb was found to be significantly higher in collagen content, would imply a systemic effect from the anabolic steroid treatment. In addition to the effect on muscle, the associated tendon has also been documented. The fibrils of tendon apparently increase in diameter measured as increased collagen content. These findings are probably associated with changes in cross-linking. The net result is a stiffer, weaker tendon which fails with less elongation. Thus anabolic steroids have a detrimental effect on the muscle-tendon unit in addition to any potentially serious systemic side effects (29).

In conclusion, it was found that after trauma, the anabolic steroid, Decadurabolin (administered in therapeutic dose), has an anabolic effect on collagen synthesis. The increase in collagen is much greater than is the increase in non-collagenous protein.

B) Electrical Stimulation

Electrical potentials stimulate the regeneration of damaged nerve and muscle structures, and accelerate surgical wound healing (11). Electrical stimulation seems to increase protein synthesis de novo, although subsequent stimulation of amino acid transport results in an additional increase in the incorporation of amino acids into proteins (9). Inasmuch as these effects only occur during the application of the electrical current, without any latent effects, electrical stimulation directly affects protein metabolism, which receives additional augmentation from the increased availability of free amino

acids (9).

In research done by Anderson and Lipscomb (2), electrical muscle stimulation did not reduce volumetric atrophy of the thigh, but it was effective in minimizing the loss of strength which occurs with immobilization. In the present study, it was found that in the group treated with electrical stimulation, there was an increase in the non-collagenous protein content of muscle compared to the group treated with an anabolic steroid, or the group which received no treatment, and significantly was also similar to the group treated with both an anabolic steroid and electrical stimulation.

Other reports have shown that when frog erythrocytes were subjected to electrical stimulation twice the amount of protein was synthesized (19). Studies have shown that fibroblasts tend to migrate towards the cathode in an electrical field (14,32). Cheng et al. (9) agreed and added that the protein content was increased, but only if both electrodes were attached. Black (8) stated that electrical stimulation increased both protein and collagen with this attraction of the fibroblasts to the cathode.

Other explanations concerning the mechanisms by which electrical stimulation enhances protein synthesis have been offered. Stanish et al. (37) suggested that electrical stimulation decreased the amount of protein breakdown which accompanied other catabolic effects in soft tissue, but that it did not have an anabolic effect. Wong and Booth (43) found that electrical stimulation increased protein synthesis, but only did so maximally if electrical stimulation was accompanied by exercise. Lastly, Eisenberg and Salmons (13) concluded that to increase protein synthesis with electrical stimulation, the process of protein synthesis and degradation needed to be sufficiently coordinated to

produce morphological changes in a continuous manner without disturbance to the structural integrity of the fibres.

In the present study, electrical stimulation did not produce a systemic effect, in as much as both groups treated with electrical stimulation showed a significant difference between the non-collagenous protein content of treated and control legs.

Karpakka et al. (24) reported that the increase in collagen concentration in the soleus muscle immobilized in a shortened position, was caused by the rapid net degradation of non-collagenous proteins. During the immobilization of the soleus muscle the total muscular weight was decreased, but the total hydroxyproline pool was unchanged (24). Bassett and Herrmann have stated that hydroxyproline content was increased by electrical stimulation (5). Electrical stimulation also has been found to modify the types of collagen produced (1). The present results showed that electrical stimulation decreased the excessive formation of collagen in the healing process.

In conclusion, the present findings illustrate that electrical stimulation increases non-collagenous protein production in the traumatized soleus muscle. This study also found that collagen production was decreased in the non-traumatized medial gastrocnemius muscle treated with electrical stimulation.

C) Anabolic Steroids and Electrical Stimulation

The results show that when the traumatized soleus muscle received treatment with both anabolic steroids and electrical stimulation, the muscle synthesized a lesser amount of non-collagenous protein than the untreated group or the group treated with electrical

stimulation alone. Nie et al. (31) used electrical stimulation as a training process and found that it increased protein breakdown sufficiently to increase the activity of the anabolic steroid given. Reviewing the previous sections in this discussion, it can be concluded that the nitrogen imbalance produced by the trauma and electrical stimulation, was not sufficient to trigger an anabolic response with anabolic steroid therapy.

In the present study, collagen content was found to be significantly decreased in the medial gastrocnemius muscle of rats treated with electrical stimulation and an anabolic steroid, compared to rats treated with an anabolic steroid alone, or rats who remained untreated.

Summary

According to the findings, electrical stimulation alone would be the recommended modality for the treatment of trauma to skeletal muscle. This recommendation stems from the findings that electrical stimulation increased non-collagenous protein synthesis, which allowed muscle to regain its contractile properties and decreased the production of collagen, which is the main component of scar tissue and which decreases the functionality of muscle.

Anabolic steroids were found to increase collagen. Although this collagen increase could be attributed to an increase in the fibroblast production in the tendinous structure, this study eliminated that possibility by lyophilising the tissue prior to analysis. The lyophilising process does not powder the tendon so it is not included during the analysis. Thus the increase in collagen found with in the muscle tissue can be attributed

to scar.

Therapeutic doses of anabolic steroids are not indicated because of 1) the potential for damage to tendinous structures and connective tissues, and 2) the lack of controlled therapeutic studies proving their usefulness in recovery from muscle injury (21, 29).

Chapter 6

Summary and Conclusions

The purpose of this study was to determine if anabolic steroids, electrical stimulation or a combination of the two modalities had an effect on collagen production as well as non-collagenous protein production in exercised rat skeletal muscle after blunt trauma. This research also set out to determine which treatment, anabolic steroids, electrical stimulation or a combination of the two treatments, had the greater effect on collagen and/or non-collagenous protein production.

A sample of thirty-seven male Sprague-Dawley rats were exercised on a treadmill for a seven week period at which point they were randomly assigned by weight to one of the experimental groups. Each rat in a treatment group received, under anaesthetic, a single impact trauma to the medial aspect of the calf of the right leg. The different treatments lasted 10 days after which the animals were sacrificed and the medial gastrocnemius muscles and the soleus muscles were excised for analysis.

The results showed that the citrate synthase levels were significantly higher in the group which had completed the training protocol. The results also showed that anabolic steroids significantly increased the production of collagen in the traumatized medial gastrocnemius muscle compared to the control limb, to the injured limb with no treatment or the injured limb treated with electrical stimulation for the homologous muscle. It was also found in this research that electrical stimulation significantly increased the amount

of non-collagenous protein compared to the group which received anabolic steroids and electrical stimulation together. The group which received anabolic steroids and electrical stimulation together had a significant decrease in non-collagenous protein content compared to the injured limb which received no treatment for the soleus muscle.

CONCLUSIONS

On the basis of the hypothesis stated in the present study, the following conclusions were made:

1) There is significant difference between the citrate synthase levels in non-exercised non-traumatized muscle and the exercised traumatized muscle.

2) There is significant difference between the collagen content in the exercised traumatized muscle untreated and the exercised traumatized muscle treated with anabolic steroids.

3) There is no significant difference between the non-collagenous protein content in the exercised traumatized muscle untreated and the exercised traumatized muscle treated with anabolic steroids.

4) There is no significant difference between the collagen content in the exercised traumatized muscle untreated and the exercised traumatized muscle treated with electrical stimulation.

5) There is no significant difference between the non-collagenous protein content in the exercised traumatized muscle untreated and the exercised traumatized muscle

treated with electrical stimulation.

6) There is no significant difference between the collagen content in the exercised traumatized muscle untreated and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

7) There is significant difference between the non-collagenous protein content in the exercised traumatized muscle untreated and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

8) There is significant difference between the collagen content in the exercised traumatized muscle treated with anabolic steroids and the exercised traumatized muscle treated with electrical stimulation.

9) There is no significant difference between the non-collagenous protein content in the exercised traumatized muscle treated with anabolic steroids and the exercised traumatized muscle treated with electrical stimulation.

10) There is no significant difference between the collagen content in the exercised traumatized muscle treated with anabolic steroids and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

11) There is no significant difference between the non-collagenous protein content in the exercised traumatized muscle treated with anabolic steroids and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

12) There is no significant difference between the collagen content in the exercised traumatized muscle treated with electrical stimulation and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

13) There is significant difference between the non-collagenous protein content in the exercised traumatized muscle treated with electrical stimulation and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

14) There is no significant difference between the collagen content in the exercised non-traumatized muscle untreated and the exercised traumatized muscle untreated.

15) There is no significant difference between the non-collagenous protein content in the exercised non-traumatized muscle untreated and the exercised traumatized muscle untreated.

16) There is significant difference between the collagen content in the exercised muscle and the exercised traumatized muscle treated with anabolic steroids.

17) There is no significant difference between the non-collagenous content in the exercised muscle and the exercised traumatized muscle treated with anabolic steroids.

18) There is no significant difference between the collagen content in the exercised muscle and the exercised traumatized muscle treated with electrical stimulation.

19) There is no significant difference between the non-collagenous protein content in the exercised muscle and the exercised traumatized muscle treated with electrical stimulation.

20) There is no significant difference between the collagen content in the exercised muscle and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

21) There is no significant difference between the non-collagenous protein content in the exercised muscle and the exercised traumatized muscle treated with anabolic steroids and electrical stimulation.

CLINICAL SIGNIFICANCE

The various modalities used in this study have been used in clinical sports medicine. Regrettably, optimal treatment times and dosages have not been standardized in the literature.

This experimental model provides a means to quantitatively assess a therapeutic modality and bring new insight to its use. However, species differences do not permit a direct application of results obtained in the rat to clinical treatment of the human athlete.

The results showed that the different modalities used in this study affect differently the different muscle groups differently, which is likely related to differences in function and metabolism of these muscle types.

In the present study, electrical stimulation treatment was found to increase non-collagenous protein production and diminish the collagen production compared to anabolic steroids treatment.

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APPENDIX 1

ANABOLIC STEROID INJECTION PROTOCOL FOR A RAT.

1) Prior to the injection, the hind limb to be treated was shaved with a regular pet hair clipper (Oster "Golden 45" 2 speed clipper, CAT. no: 5-61).

2) Experimenter 1 removes the rat from the cage and holds the rat in one hand while extending the untreated limb (left) with the other hand.

3) Experimenter 2 aspirates, with a 22 gauge needle, the dosage of Decadurabolin (5mg) into the syringe (1ml).

4) Experimenter 2 assures that there are no air bubbles in the content of Decadurabolin in the syringe.

5) Experimenter 2 changes the 22 gauge needle for a 27 gauge needle on the 1ml syringe.

6) Experimenter 2 extends the treated limb (right) with one hand while holding the syringe in the other hand.

7) Experimenter 2 injects the dosage of Decadurabolin into the medial aspect of the right calf.

8) Experimenter 2 withdraws the needle from the medial aspect of the right limb of the rat and releases the limb.

9) Experimenter 1 puts the animal back in its cage.

APPENDIX 2

ELECTRICAL STIMULATION PROTOCOL FOR A RAT.

1) Prior to electrical stimulation treatment, the hind limb to be treated was shaved with a regular pet hair clipper. (Oster "Golden 45" 2 speed clipper, CAT. no:5-61).
2) Experimenter 1 removes the rat from the cage and holds the rat in one hand while extending the treated limb (right) with the other hand over a support sponge.
3) Experimenter 2 covers the rat's head with a towel thus the animal is in the dark during the treatment reducing anxiety to the animal.
4) Experimenter 2 places the electrodes of channel 1 on the rat's extended leg. The red electrode (anode) at the distal end of the gastrocnemius and the white electrode (cathode) in the inguinal region over the femoral nerve. Both electrodes are covered in gel which allows conduction of the current and through the skin.
5) The "Focus" electrical stimulator is set a 50 pulse rate. The duty cycle will be set at 5 seconds on and 5 seconds off, using a monophasic wave.
6) Experimenter 2 increases the current output of channel 1, in a continuous mode, until a tetanic contraction occurs.
7) Experimenter 2 then changes the continuous current output mode, to an on/off duty cycle.
8) The treatment time will be 5 minutes from this point on.
9) After the 5 minute treatment is completed, experimenter 2 turns off the current output and removes the electrodes from the rat then experimenter 1 puts the animal back in its cage.

APPENDIX 3

BIOCHEMICAL ANALYSIS

Method of Woessner (1)

Reagents:

1. Citrate buffer

   50g (citric acid.H2O) + 120g Sodium Acetate.3H2O + 34g NaOH + 12ml of glacial acetic acid dilute to 1 liter with DH2O. Adjust pH to 6.00 and refrigerate. (Shelf life = 1 month).

2. Chloramine T (Fresh daily)

   0,4g chloramine T (Kodak) + 2 ml of DH2O + 3ml of methyl cellosolve + 5ml of citrate buffer.

3. Perchloric Acid (19% v/v)

   Stock Percloric Acid is 70%.

4. P-Dimethylamino benzaldehyde (P-DMABA).

   Fresh daily (20g P-DMABA in 100 ml cellosolve)

   Heat to dissolve (60 C).

Protocol:

1. Prepare a stock solution of 30ug/ml of L-Hydroxyproline (Sigma).

2. Pipet 2ml of the standard containing 1-5ug of L-Hydroxyproline.

3. At timed intervals add 1ml of chloraime T and Vortex. Allow oxidation to take place for 20 minutes at room temperature.
4. At timed intervals add 1ml of perchlorate solution at precisely 20 minutes. Stop oxidation and let samples stand at room temperature no greater than five minutes.
5. Add 1ml of P-DMABA and mix well.

   Incubate in tank at 60 C for maximum Chromophore development (20 minutes).
6. Allow samples to equilibrate to room temperature and mix before reading at 557nm.

Tissues samples are hydrolysed in 6MHCl at 105 C for 24 hours. 1mg collagen or tissue to 2mg of 6MHCl. Remove acid after hydrolysis in vacuum. Redissolve samples in DH2O.

Lowry's Non-Collagenous Protein Determination (4)

Reagents:

A. i. 5% cupric sulfate (CuSO4)

   ii. 10% sodium tartrate (fresh) 50mg/.5ml DH20

   iii. 10% sodium carbonate in 2% NaOH (stock)

B. i. 1ml Folin

   ii. 1ml DH2O 50% Solution

C. Protein standard stock solution: 1mg/ml BSA in H2O.

Protocol:

Take sample volume up to 0.5ml.

i. Add 0.1ml A 10 minutes EXACTLY

ii. Add 0.05ml B 30 minutes

Spectrophotometric analysis

i. Set spectrophotometer wavelength to 750nm.

ii. Read and record optical density of each sample.

iii. Plot standard curve (x=Protein concentration, y=O.D.) and estimate unknown protein concentrations.

Citrate Synthase Assay (5)

Reagents:

Homogenizing Medium (500ml)

i. 50mM Tris-HCl.

ii. Draw 100ml and pH to 8.1.

iii. To the rest add 1mM EDTA and then 0,4g Triton X-100 then pH to 7,6.

Reaction Medium

i. Make stock solution of DTNB 1mM.

ii. Add 1ml of DTNB stock to 9ml Tris-HCl.

iii. Add 2,84 mg of Acetyl-Co-A to DTNB and Tris-HCl Mix.

Oxaloacetate

i. Make solution and divide into 100ul aliquots to use for each batch of 16 assays.

ii. Store at -70C and thaw as needed.

iii. Use 5ul/assay of 13,2 oxaloacetate/ 1ml of 100mM Tris-Hcl buffer.

Protocol:

For optimal activity

i. Pre-chill grinder and cuvettes.

ii. Homogenize gently in glass tissue grinder.

iii. Sonicate (3x5 sec) with 30 sec intervals.

iv. Keep everything on ice.

v. Pre-chill centrifuge at 4C.

vi. Centrifuge @ 12000xg for 5 min.

vii. Use plastic cuvettes.

Reaction Assay in spec cuvette

i. 5ul supranatant sample.

ii. 590ul stock.

iii. 5ul oxaloacetate to start reaction.

Spectrophotometer settings

i. 412nm.

ii. span 0,5A.

iii. off 0,3A.

iv. speed 10s/cm

APPENDIX 4

TRAINING PROTOCOL (No: 90147)

Week	Duration(min)	Grade%	Speed(m/min)	Number of Sessions/day
1	10	0	10	2
2	15	0	15	2
3	20	0	20	2
4	25	2	25	2
5	25	2	25	2
6	30	2	25	2
7	30	2	25	2

APPENDIX 5

Current Output from the Electrical Stimulator.

(Focus NMS unit, ANSI/AAMI NS4-1985)

The current output of the Focus unit was measured before and after this study at the Clinical Engineering Department of the University of Alberta Hospital. To obtain these measurements, a circuit was set up.

A 0,1 microfarad capacitor was placed in parallel with a 1 ohm resistance, an oscilloscope and the Focus unit. See Figure 7.

The oscilloscope was thus situated to be able to measure the current which came through the circuit once the electrical stimulator was turned to it's different settings.

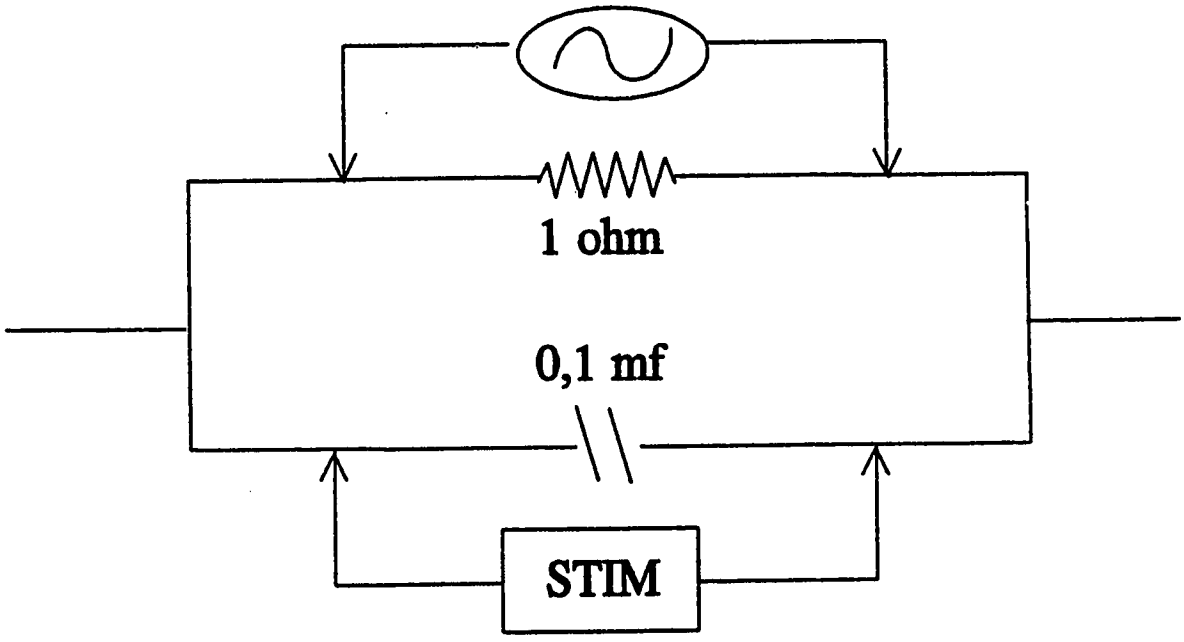

Figure 7: Circuit testing current output of Focus NMS unit.

The capacitor represented the rat and the resistor represented the skin impedance.

The current output values before and after this study were almost identical thus it can be said that the current output during the experiment remained the same. See Table 16.

	Current Output	
Settings	pre-experiment	post-experiment
10	13 mA	9,5 mA
20	30 mA	30 mA
40	50 mA	48 mA
60	65 mA	64 mA
80	90 mA	85 mA
100	100 mA	93 mA

Table 16: Current output of Focus NMS unit.

It should be noted that the post-experiment measures were made with the original batterie. This could explain the decrease in current output at the 80-100 settings in the post-experiment evaluation.