HISTOLOGY, HISTOCHEMISTRY AND MORPHOMETERY OF OVERLOADED SKELETAL MUSCLES UNDER NORMAL, HYPO AND HYPER-THYROID STATUS N PIGEON

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Abstract: The plantaris muscle in both legs of each experimental animal was overloaded through tenotomy of its synergistic gastrocnemius muscle. The characteristic response pattern of overload involved increase in wet muscular weight, hypertrophy, increase in Average Cross-sectional Area (AFCA) of the muscle fibers and a trend of shift from glycolytic and intermediate fibers towards oxidative fibers. All these basic responses are initially augmented in hyperthyroid situation. Under prolonged (5-10 days) exposure to T4 the overloaded muscles showed degenerative changes such as decline in muscular weight, atrophy and decrease in AFCA while the fiber shifting process continues without any major modification. Fibrosis was another important effect of prolonged exposure to thyroxin. Treatment with thiourea decreased the release of T4. Responses of muscular overload in hypothyroid situation include gradual increase in muscular weight, an increase in AFCA up to 10 days stage, and a progressive shift from glycolytic and oxidative fibers to intimidate fibers. A decrease in AFCA (an indicative of secondary atrophy in individual muscle fibers) with a concomitant increase in fibrosis was noted at 15 days stage in this experimental group. Conclusively, it is suggested that thyroxin enhances the anabolic and oxidative potentials of skeletal muscles under the stress of overload, but prolonged stress under hyperthyroid conditions lead to degenerative changes; on the other hand, hypothyroid situations lead to progressive muscular loss in the same stress condition.
Keywords: Histochemistry, morphology, overload, skeletal muscle, thyroidism, tenotomy, and pigeon.

INTRODUCTION

keletal muscles are especially targeted by the thyroid hormones. Brown and Millward (1983) studied dose response of protein turnover in rat skeletal muscle to tri-iodothyronine treatment. They concluded that protein synthesis and degradation were generally lower in the hypothyroid state and normal or elevated in the hyperthyroid state. The increased protein turnover in the hyperthyroid rats is argued that this is necessary to allow the changes in protein composition and metabolic character which occur in response to hyperthyroidism. Experimental hyperthyroidism reduces skeletal

muscle mass. Increased muscle protein degradation may be a major factor in the development of skeletal muscle wasting and weakness in hyperthyroidism. Hyperthyroidism caused a 25-29% increase in protein breakdown in both sarcoplasmic and myofibrillar fractions of skeletal muscle (William *et al.,* 1980). Chronic hyperthyroidism reduces N-contents of muscles, urinary urea-N excretion and N-balance. Thyroid hormones thus mobilize muscle-N (Grofte *et al.,* 1997). Where as chronic alterations in thyroid status specially affect oxidative damage to lipids in skeletal muscle, with a probably stronger effect on mitochondria! membranes, whereas the cytosolic redox potential and DNA are better protected possibly due to homeostatic compensatory reactions (Gredilla *et al.,* 2001). The changes in physiologic properties and fiber type composition are generated by a direct action of thyroid hormone on muscle fibers (Mtintener *et al.,* 1987).

Uncoupling proteins (UCP) has been recently demonstrated to be strongly regulated by thyroid status in chicken, and over expressed in experimental conditions favoring high tri-iodothyronine concentrations and thermogenesis. However, its real uncoupling activity and contribution to thermogenesis remain to be established (Collin *et al.,* 2005). Thyroid hormones stimulate UCP-3 expression in skeletal muscles. The effect of T_3 on UCP-3 expression in cardiac and skeletal muscle is not dependent on either angiotensin II or the β-adrenergic system and probably reflects a direct action of the hormone on UCP-3 gene expression (Silva *et al.,* 2004). The expression of avian Hyperthyroidism was found associated with an increased leakage of protons through the inner mitochondrial membrane, whereas the opposite occurs in hypothyroid mitochondria It is tempting to speculate that thyroid hormones also alter calcium concentration and thus influence the process of excitation-contraction coupling in the skeletal muscle (Hudecova *et al.,* 2004).

Hypothyroid dogs tend to develop hyperkalemia during exercise, which for a large part can be explained by the severe reduction of the Na^+-, K^+-ATPase capacity in the skeletal muscle pool (Schaafsma *et al.,* 2002).

In a study primary chick muscle cells were treated with physiological level of thyroxin (T_4) or tri-iodothyronine (T_3) to examine the effects of the hormones on growth, protein turnover, and apoptosis of the cells. Creatine kinase activity, as an index of differentiation, was increased by both T_4 and T_3. The rate of protein degradation estimated from [^{3}H] tyrosine release was increased by T_3 but not by T_4. DNA cleavage and fragmentation, as indices of apoptosis, were induced by T_3 but not by T_4. These results show that T_4 stimulates cell differentiation but not protein degradation and apoptosis in primary chick muscle cells, while all events are stimulated by T_3 (Nakashima *et al.,* 1998).

Most prominent load associated change in skeletal muscle is hypertrophy. The metabolic aspect leading to skeletal muscle hypertrophy is the increase in protein synthesis mainly attributable to increased mRNA activity. Muscle growth follows when a

positive protein balance is established and maintained by an increase in protein synthesis that exceeds the rate of protein breakdown (Pitkanen *et al.,* 2003). The cellular aspect of hypertrophy involves an increase in mRNA abundance due to an increased transcriptional activity via differentiation and proliferation of satellite cells. The differentiating satellite cells donate additional myonuclei to the enlarging muscle fibers (Adams and Haddad, 1996). Unlike most other cells, skeletal muscle fibers are multinucleated. Each and every nucleus is responsible for a particular volume of the cell, known as a *nuclear domain* (Fry, 2004). This domain is tightly regulated and any increase in the fiber cross-sectional area requires a concomitant increase in the number of myonuclei (Aliens *et al.,* 1996).

The concept of a finite relationship between fiber size and myonuclei number predicts that the hypertrophying fibers must increase their myonuclear number proportionally. However, shortly after birth, mammalian myofibers are permanently differentiated, and thus cannot undergo mitotic division or directly increase their myonuclear number by means of the usual myonuclear division process (Chambers and McDermott, 1996). Therefore, hypertrophying fibers require an external source of new nuclei to maintain a relatively constant nucleus-to-fiber size ratio. A significant body of evidence blames satellite (stem) cells as the probable source of the new myonuclei (Barton-Davis, 1999; Sinha-Hikim, 2003). The role of satellite cells in hypertrophy has been further corroborated by studies using radiation to prevent satellite cell activity, thereby negating any potential hypertrophic response (Phelan and Gonyea, 1997; Adams, 2002).

Above mentioned literature clearly indicates the load associated anabolic changes and the importance of thyroxin in muscular metabolism. Unfortunately the available literature does not throw any light on the effects of these two factors combined. It was thus decided to investigate the effects of over-load on the skeletal muscles under hyperthyroid, euthyroid and hypothyroid conditions, in avian system.

MATERIALS AND METHODS

This study was carried on male pigeons. Thirty-six animals approximately 6 months of age, weighing 300-350 grams, were used. They were divided into four groups of nine animals each; namely Control (C) Group, Vehicle Control (VC) group, Thyroxin (T4) group and Thiourea (TU) group. Plantaris muscle in both legs of animals of VC, T4 and TU groups was overloaded by tenotomising its synergist the "gastrocnemius muscle". Control animals were sham operated in which a longitudinal cut was given to fully expose the gastrocnemius muscle on both legs; the incisions were sutured back with out damaging the under-laying muscles and tendons.

Procedure of tenotomy

Anesthetic ether was used to anesthetize the Animals. Feathers were carefully removed from the back portion of the shank area on both legs. By giving longitudinal

incisions the gastrocnemius muscles were exposed. Achilles tendon in each leg was carefully isolated from the adjoining tissues and then snipped with sharp scissors nearest to its insertion. Using appropriate sterilized suturing needle, the cut stump of the tendon was sutured at the base of the proximal end of the muscle on the underside of the skin flap in such away that the cut end faced away from its point of insertion. Finally the skin was sutured back with "4-O" suturing thread.

Dose preparations and applications

Through dilutions in distilled water, all doses were prepared in such a way that 1.0 ml of the solution should contain the required dose amount. These doses were delivered on daily bases, to the experimental animals in the gullet carefully with the help of an appropriate syringe. Needle of the syringe was covered with a rubber tubing to avoid any oral injury to the animals. Pigeons in VC group received 1.0 ml distilled water. T4 group pigeons received 0.5 mg/kg BW (body weight) Thyroxin and the TU group animals received 5 mg/kg BW thiourea.

Recovery

Three animals were recovered from each group, on 5th, 10th, and 15th day. In the recovery process each animal was again anesthetized. Gastrocnemius muscle were completely detached from both legs in order to fully expose the under laying plantaris muscles. Complete belly of plantaris muscle from each leg was then carefully removed weighed and placed in Bouin's fixative for 24 hours.

Histology and histochemistry

The muscles were processed for wax imbedding in a routine way, sectioned at 6 micron using rotary microtome and stretched on glass slides. Hematoxylin and Eosin staining method was used for histological and morphometric studies. Three slides per muscle were stained for the presence of glycogen in the muscle fibers with PAS-shiff reagent. On the bases of relative PAS staining properties muscle fibers were categorized into the following three groups; Glycolytic fibers (PAS^+), Intermediate fibers and Oxidative fibers (PAS^-). To show the relative abundance of these three types the muscle fibers were counted directly on MARUZEN 300 microscope projector.

Morphometery

Camera lucida drawings of the muscle fibers were used for morphometric analysis. For this purpose five randomly selected areas from six different sections were drawn on plain paper from each such muscle at 400X. The cross-sectional area of each muscle fiber was calculated using polar planimeter (Kt-type). The planimeter readings were converted into measurements in micron meter square *$(\mu m)^2$* with the help of following formula.

$$\text{Area } (\mu m)^2 = \text{planimeter reading X } (10)^6/(\text{Magnification})^2$$

From the data generated average muscle fiber size values were obtained for control and each experimental group. To show the response patterns in different groups the average values of weight and muscle fiber sizes were expressed in graphs.

RESULTS

Morphology and histology

Following overload a general trend towards increase in weight and AFCA of plantaris muscle was observed. Initially at 5 days stage trend towards increase in muscular weight and AFCA was further enhanced under hyperthyroid condition. However, at later stages i.e. 10 and 15 days a secondary atrophy was observed as indicated by decreased muscular weight and AFCA (Fig. 7 and 8). At the same time a general trend towards the development of connective tissue fibers was clearly observed (Fig. 4). In hypothyroid condition a more gradual but slightly enhance trend of increase in muscular weight was observed. A similar trend towards increase in AFCA was seen up to 10days stage followed by the secondary atrophy leading to decline in AFCA (Fig. 7 and 8). The development of connective tissue seems to be following a similar pattern as in the hyperthyroid condition (Fig. 5 and 6).

Histochemistry

Plantaris appears to be a mixed muscle. Generally simple overload induces an increase in percent number of oxidative fibers, mostly to the detriment of Intermediate fibers. With a slight initial increase in intermediate fibers (at 5 days stage) at the expense of both the glycolytic and oxidative fibers the VC group shows a consistent increase in oxidative fibers mostly at the cost of intermediate fibers (Fig. 9). A similar but slightly modified response towards fiber conversion was seen in hyperthyroid condition. In T4 group the initial rate of increase in percentage of oxidative fibers was far greater than VC group. Moreover this conversion seems to be drawn from intermediate fibers only. At 10 days stage oxidative fibers increased in a more gentle way simultaneously the number of intermediate fiber increased definitely at the expense of glycolytic fibers. At 15 days stage oxidative fibers increased but at this stage completely at the cost of intermediate fibers (Fig. 10). In hypothyroid condition at 5 days stage only slight modification to that of control was seen in the percent number of three types of fibers. At the later two stages however the number of intermediate fibers increased more consistently at the cost of both the oxidative and intermediate fibers (Fig. 11).

DISCUSSION

Although most of the studies in this regard have been confined to mammalian system; in general prospective overload brings above hypertrophy and increase in the muscular weight (Goldspink, 1977; Mufti and Qureshi, 1989). Morphometric analysis on the basis of AFCA of the muscle fibers gives a clear indication that AFCA continues to increase with increasing duration of overload. We can clearly speculate that overload brings about anabolic changes in skeletal muscles such as increase in protein deposition. Consequently overloaded skeletal muscle shows an increase in muscular weight, hypertrophy and increase in AFCA.
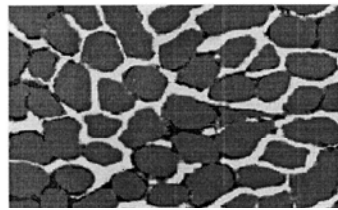
Fig # 1: Plantaris Control   Fig # 2: 15 days overload (CV. Group)
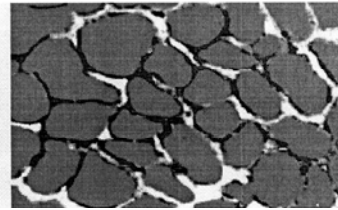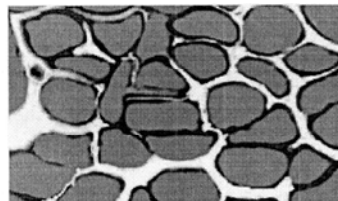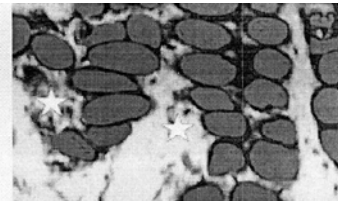
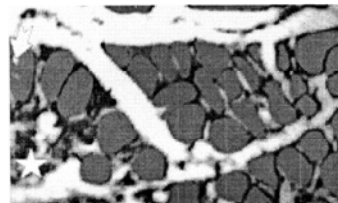Fig # 3: 10 days overload (T4 Group)   Fig # 4: 15 days overload (T4 Group)

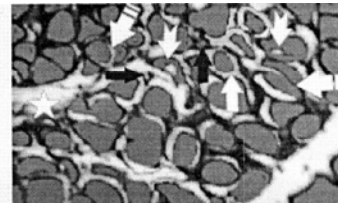Fig # 5: 10 days overload (TU Group)   Fig # 6: 15 days overload (TU Group)

Fig. 1: Note the compact fiber arrangement; Fig. 2: Note a general hypertrophy of the muscle fibers with compact arrangement; Fig. 3: Slightly shrunken muscle fibers; Fig. 4: An obvious shrinkage in muscle fibers with a great deal of fibrosis (White Stars); Fig. 5: Development of fibrotic mass at the expense of muscle fibers reduction (White star), blue arrow shows the central core lesion; Fig. 6: Note: Fibrosis (White star), central core lesions (Notched arrow), shrinkage of individual fibers (White arrow), splitting muscle fibers (Broken arrows) and individual necroting fiber (black arrow).
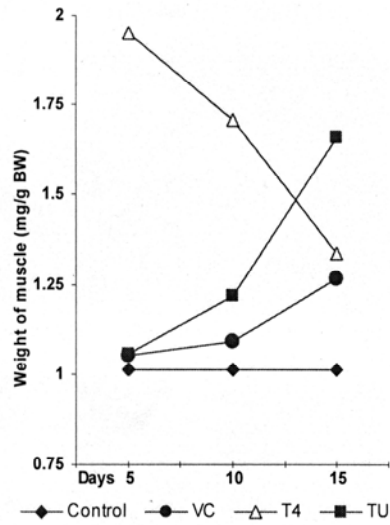
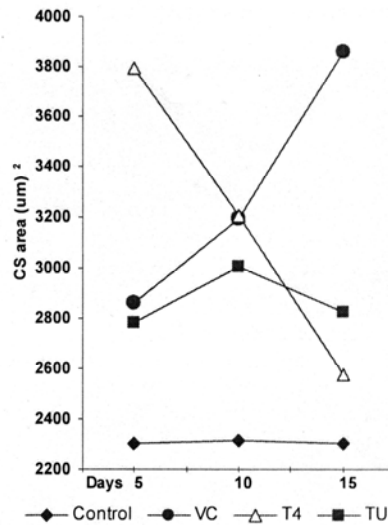**Fig.7. Comparative weight response of plantaris muscle in three experimental conditions with control**
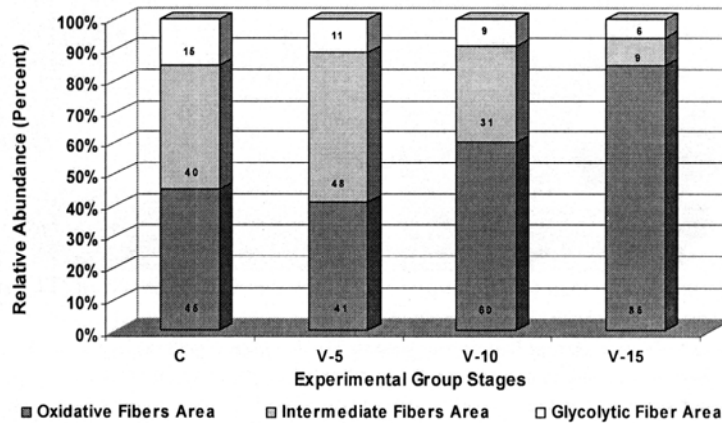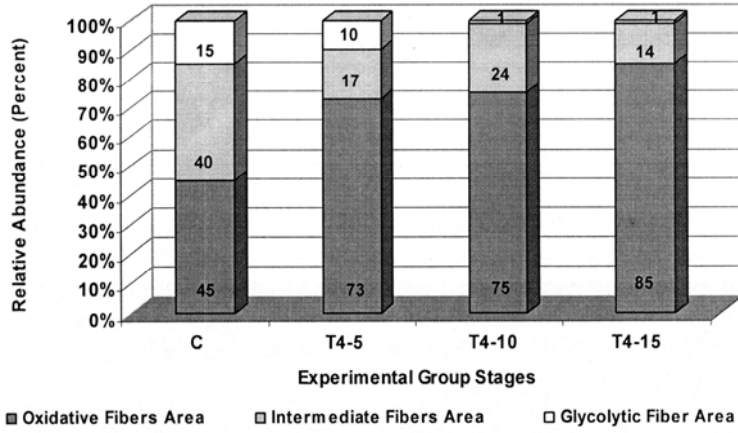

Fig.8. Comparison of average CS area of Plantaris muscle fibers in three experimental conditions with control
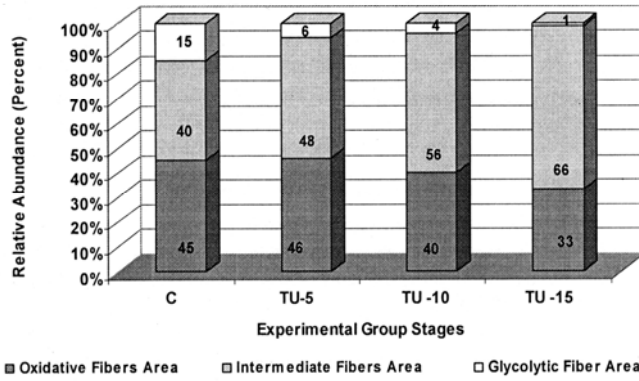

C: Control; V-5: 5days Overload; V-10: 10days Overload; V-15: 15days Overload
Fig.9. A graph showing relative abundance of three types of muscle fibers at different durations of overload under euthyroid condition in comparison with control

C: Control; T4-5: 5days Overload in hyperthyroid condition; T4-10: 10days Overload in hyperthyroid condition; T4-15: 15days Overload in hyperthyroid condition

Fig. 10. A graph showing relative abundance of three types of muscle fibers at different durations of overload under hyperthyroid condition in comparison with control

C: Control; TU-5: 5days Overload in hypothyroid condition; TU-10: 10days Overload in hypothyroid condition; TU-15: 15days Overload in hypothyroid condition

Fig 11: A graph showing relative abundance of three types of muscle fibers at different durations of overload under hyperthyroid condition in comparison with control

Under hyperthyroid situations maximum weight gain and increase in AFCA was observed at 5 days stage followed by a decreasing trend in these two factors in the later stages. The most obvious explanation in this connection maybe that continuous exposure toT4 possibly brings about catabolic changes resulting into a gradual atrophy in this case. Histological findings support this explanation where we see a gradual wastage of the muscular mass along with increased fibrosis (Fig. 4).

In hypothyroid condition the muscular weight response followed the similar but slightly enhanced pattern as that of the control condition; however AFCA kept on increasing up to 10 days stage afterwards it shows a clear decline. The logical reason for this pattern of response may be the accumulation of interstitial fluid as we do see necrosis leading to shrinkage of muscle fibers with a little fibrosis in between (Fig. 6).

A continuous shift of glycolytic and intermediate fibers to oxidative fibers with the increasing duration of overload clearly indicates that overload obviously increase the oxidative capacity of skeletal muscles. Thyroxin being an enhancer of oxidative metabolic activities has a positive impact on this fiber shifting process. Interesting alterations in the fiber shifting process under hypothyroid condition again indicate the possible role of thyroid status. We see the general trend of increase in the intermediate fibers at the cost of both glycolytic and oxidative fibers with the increasing duration of overload. Overload must be playing a very important role in the conversion of glycolytic fibers to intermediate fibers at the same time hypothyroid situation may be leading to a decrease in the oxidative capacity of purely oxidative fibers. This is because we see a continuous increase in the number of intermediate fibers at the expense of glycolytic and oxidative fibers.

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