AMELIORATION OF STRESS INDUCED DAMAGE IN MICE GASTROCNEMIUS BY FENOTEROL HYDROBROMIDE

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ABSTRACT
Treatment of normal innervated mice with fenoterol resulted in significant increase (10.85%) in body weight at day 28. Fenoterol treatment to denervated mice showed 5.15% increase in body weight at day 7 and 3.80% at day 28. Moreover, fenoterol treatment resulted in amelioration of muscle atrophy at all the stages and there was a significant increase (4.91%) at day 28 in denervated fenoterol treated animals. There was a constant increase in gastrocnemius dry mass in fenoterol treated mice. However, fenoterol treatment increased the dry gastrocnemius mass at all the stages of investigation in denervated animals. The effects of fenoterol on denervated mice healed the effects of denervation but its value was significantly below the normal innervated levels. Histopathological examination of denervated mice treated with fenoterol showed ameliorating effects in gastrocnemius muscle which showed some recovery of muscle structure towards normal profile.

KEY WORDS: Mice, Gastrocnemius, Hypertrophy, Atrophy, Denervation, Fenoterol

INTRODUCTION
A progressive loss of skeletal muscle mass (sarcopenia) and a subsequent decline in muscle strength are the starting symptoms of ageing (Larsson and Ramamurthy, 2000; Morley et al 2001). Over the past two decades much research has been focused on the underlying mechanisms of age-related effects on skeletal muscle, responsible for the gradual loss of functional independence amongst the elderly. Progressive muscle fibre denervation, a loss of motor units, and potential motor unit remodelling have been implicated, but since the slowing of contraction occurs before significant muscle wasting, intrinsic changes to skeletal muscle fibres, including excitation–contraction coupling, cannot be ruled out (Faulkner et al 1995; Larsson, 1995; Plant and Lynch 2002; Lynch and Ryall, 2008). Systemic administration of β-agonists can improve skeletal muscle regeneration after injury. However, therapeutic application of β-agonists for muscle injury has been limited by detrimental cardiovascular side effects. Intramuscular agonist administration may obviate some of these side effects (Ryall et al 2008). Pharmacological agents that promote muscle protein accretion have clinical potential for improving muscle regeneration after injury. Synthetic β2-adrenoceptor agonists, such as fenoterol and clenbuterol, were initially developed for acute asthma treatment, to facilitate bronchiolar smooth muscle dilation (Van Nolte et al 1974). The β2-agonist fenoterol has potent anabolic effects on rat skeletal muscle. Muscle mass and force-producing capacity of skeletal muscle has been attributed to a non-selective increase in the cross-sectional area of all muscle fibre types. Fenoterol treatment causes a small increase in fatigability due to a decrease in oxidative metabolism in skeletal muscle fibers (Larsson and Ramamurthy 2000; Ryall et al. 2004). Administration of a β2-adrenoceptor agonist has profound effect on global gene expression in skeletal muscle. β2-adrenoceptor agonist treatment alters the expression of several genes associated with myostatin signaling during hypertrophy (Pearin et al. 2009). Despite their muscle anabolic properties, β-agonists have also been associated with some undesirable side effects, including increased heart rate and muscle tremor, which have so far limited their therapeutic potential. Therefore, present study aimed at effects of β-adrenergic stimulation using fenoterol and its effects on structure and functions of skeletal muscles have been explored in detail. Even though fenoterol induced muscle specific remodeling increased the muscle performance in skeletal muscle. A greater understanding of β-adrenergic signaling in skeletal muscle is important for identifying its role in muscle growth, development and regeneration, and for identifying new therapeutic targets. Much more research is needed to understand how the β-adrenergic signaling pathway can be manipulated for the purpose of attenuating the muscle wasting associated with many diseased conditions, and enhancing muscle fiber growth, repair and regeneration. Therefore, amelioration of stress induced damage in mice gastrocnemius muscle by fenoterol hydrobromide was investigated.

METHODOLOGY
The present investigation was conducted on adult swiss albino mice of Balb-C strain weighing 24-26 g, procured from Central Research Institute (CRI), Kasauli, Himachal Pradesh. These were maintained in the animal house of the department of Biosciences, Himachal Pradesh University, Shimla under suitable hygienic conditions for 28 days experimental period. Normal healthy looking mice showing no sign of morbidity were divided into four groups, as, i) control animals, ii) drug
treated innervated animals, iii) denervated animals, iv) drug treated denervated animals. Standardized daily dose of 1.4 mg/kg body weight of fenoterol hydrobromide was given to group (ii) and (iv) for 28 days. Mice were killed by cervical dislocation at intervals of 7, 14, 21 and 28 days.

i) Drug administration and tissue harvesting
A stock solution of fenoterol hydrobromide (2 mg/ml) was prepared in distilled water. Second and fourth groups of mice were administered with chronic oral dose of 1.4 mg/kg body weight of fenoterol hydrobromide. Body weight of animals was recorded every week for 28 days. Gastrocnemius muscle was immediately excised and weighed for further experimental proceedings.

ii) Dry muscle mass and total tissue proteins
Dry muscle mass of mice gastrocnemius muscle was calculated according to Heverberg et al. 1975 and Agrawal et al. 2003. A weighed amount of tissue was homogenized in nine volumes of ice-cold distilled water. Proteins were precipitated in 10% TCA at 4°C for 15 min. Precipitated proteins were separated by centrifugation at 2000 x g for 20 min and washed twice with ice-cold 10% TCA. The precipitate was then washed successively in (i) ice-cold 95% ethanol, (ii) ethanol: chloroform = 3: 1, (iii) ethanol: ether = 3: 1 (twice), and (iv) ether. The residue was dried to a constant weight in vacuum-drying oven. The dried powder was then weighed to determine the dry muscle mass. Total protein of muscle homogenate was estimated according to Lowry et al. (1951).

iii) Histological studies
Haematoxylin-eosin staining procedure was employed to study the histopathological changes in the tissues. Immediately after sacrificing the animals, tissues were excised, cut into small pieces of 4-5 mm thickness and fixed in Bouin’s fixative for 24 hrs. These tissues were washed in running water until the entire yellow colour disappeared. The tissues were dehydrated serially from 30% alcohol to absolute alcohol, cleared in xylene and embedded in paraffin wax (58-60°C). Sections of about 6 µm thickness were cut on the rotary microtome and subjected to Haematoxylin-eosin staining.

Ribbons of the cut sections were stretched on the albuminized slides and dewaxed in xylene at 37°C overnight. These sections were then hydrated through descending alcohol grades from absolute to 30% alcohol (5 minutes each) and finally in distilled water. Sections were stained in Delafield’s Haematoxylin for 30 minutes. Differentiation was achieved in 0.1% ammonia water. Dehydration of the tissues was done by passing the sections through ascending grades of alcohol (30% to 90%; 10 minutes each). Counter staining was achieved in 1% eosin (2 minutes). Excessive stain was removed in 90 % alcohol. Sections were then dehydrated in absolute alcohol giving two changes of 10 minutes each, cleared in xylene and mounted in DPX for permanent storage. Slides were dried, examined under Leica photoscope and photographed.

RESULTS

i) Body weight, dry muscle mass and total tissue protein
Treatment of normal innervated mice with fenoterol resulted in significant increase (10.85%) in body weight at day 28. Fenoterol treatment to denervated mice showed 5.15% increase in body weight at day 7 and 3.80% at day 28 (Table 1). Moreover, fenoterol treatment resulted in amelioration of gastrocnemius muscle atrophy at all the stages and there was a significant increase (4.91%) at day 28 in denervated fenoterol treated animals (Table 2). There was a constant increase in gastrocnemius dry mass in fenoterol treated mice. However, fenoterol treatment increased the dry gastrocnemius mass at all the stages of investigation in denervated animals (Table 3). The effects of fenoterol on denervated mice healed the effects of denervation but its value was significantly below the normal innervated levels.

<table>
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<tr>
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<th>14</th>
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<td>N</td>
<td>26.2±1.32</td>
<td>27.7±1.37</td>
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<td>27.9±1.51*</td>
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<td>6.48</td>
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<tr>
<td>DnF</td>
<td>26.5±1.49*</td>
<td>27.5±1.45*</td>
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<td>5.15</td>
<td>4.56</td>
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N-normal; F-fenoterol; Dn-denervated; DnF-denervated+fenoterol

Total tissue proteins in gastrocnemius muscle showed an increasing trend after daily fenoterol treatment. A significant increase of 9.92% was reported in innervated treated animals at day 28. Moreover, there was a significant and constant decrease in total tissue proteins of denervated mice at all the stages of investigation (7 to 28 days), which has been attributed to denervation induced muscle atrophy. During the present investigation this muscle atrophy has been alleviated by daily fenoterol administration to denervated mice, as there was a significant increase of 15.14% at day 28 (Table 4).

TABLE 2: Changes in gastrocnemius muscle to body weight ratio (mg/g) after administration of fenoterol hydrobromide from 7-28 days. Values are presented as mean ± SEM; *p<0.05 (n>8).
### TABLE 3: Changes in dry muscle mass of gastrocnemius (µg/mg fresh tissue weight) after administration of fenoterol hydrobromide from 7-28 days. Values are presented as mean ± SEM; *p<0.05 (n>8).

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**% increase**

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### TABLE 4: Changes in total tissue proteins of gastrocnemius muscle (µg/mg fresh tissue weight) after administration of fenoterol hydrobromide from 7-28 days. Values are presented as mean ± SEM; *p<0.05 (n>8).

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**% increase**

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### ii) Histopathological examination

Cells of gastrocnemius fibers exhibited polygonal, circular or oval shapes with subsarcolemmal disposition of nuclei in normal innervated muscles. Muscle section showed oval shaped and vesicular nuclei. Constituent muscle fibers displayed variable shapes and sizes and hence point towards a heterogeneous population of cells (Plate 1 A).

Muscle section exhibited some hypertrophied fibers with PMNL infiltration in interfascicular spaces around blood vessels after 7 days of fenoterol administration (Plate 1 B). Adrenergic effects of fenoterol were enhanced at day 14 as shown by the presence of enormously hypertrophied fibers (Plate 1 C). Hypertrophy accompanied by merging of constituent muscle fibers was reported at day 21.

Moreover, PMNL infiltration was also seen around interfascicular spaces (Plate 1 D). Denervation of muscle led to the onset of dedifferentiation due to lack of neural trophic factors essential for the normal growth, development and other related processes. It begins with disfiguration of constituent fibers as exhibited by atrophied myofibrils with decreased inter-myofiber spaces and PMNL infiltration, as reported at day 7 of denervation. Myodegeneration characterized by the appearance of central foci of degeneration were observed. It was further accompanied by migration of muscle nuclei from subsarcolemmal or sarcolemmal disposition to central loci (Plate 2 A). Shape and size of nuclei changed to a large extent associated with disfigured fibers at day 14 of denervation. Some of the muscle cells were demonstrating transverse fissures or breaks and the fibers were likely to undergo splitting. Many muscle cells were commonly undergoing necrosis in the area adjoining the long streaks of PMNL cells. The invariable association between PMNL and degenerating muscle cells suggests that the former release substance most likely hydrolases or other proteolytic enzymes which stimulate myodegeneration (Plate 2 B). Complete atrophy of fasciculi associated with missing of interfascicular spaces was reported at day 21. Moreover, heavy infiltration of PMNL among muscle cells suggested the atrophy of fibers due to lack of normal neurotrophic regulation of muscles (Plate 2 C). Disfigurement of cells reached its peak at day 28, associated with atrophy of myofibers and heavy PMNL and macrophages infiltration to scavenge degenerating fibers (Plate 2 D).

**Plate-1**
Stress induced damage in mice by fenoterol hydrobromide

Transverse section (T.S.) of gastrocnemius muscle from normal innervated mice depicting cells with circular, oval or polygonal shapes and nuclei aligned along subsarcolemmal regions X 200.

T.S. of gastrocnemius muscle from innervated fenoterol treated mice at day 7, demonstrating hypertrophied fibers (↑) with PMNL infiltration in the interfascicular spaces and around blood vessels (↑↑) X 400.

T.S. of gastrocnemius muscle from innervated fenoterol treated mice at day 14, exhibiting enormously hypertrophied fibers (↑). Shape changes are noticed in the muscle fibers (↑↑) and some fibers showing myofibrillar degeneration (↑↑) X 400.

T.S. of gastrocnemius muscle from innervated fenoterol treated mice at day 21, revealing merging of fibers (↑) with PMNL infiltration in the muscle cell spaces (↑↑) X 400.

Plate-2

T.S. of gastrocnemius muscle from denervated mice at day 7, depicting atrophied myofibers with decrease in intermyofibrillar spaces (↑), PMNL infiltrations around myofibrils (↑↑) and some of the nuclei shifted to central position (↑↑) X 200.

T.S. of gastrocnemius muscle from denervated mice at day 14, exhibiting disfigured muscle cells (↑) with variously shaped nuclei (↑↑) X 200.
T.S. of gastrocnemius muscle from denervated mice at day 21, demonstrating heavy PMNL infiltration among muscle cells (↑), missing of interfascicular spaces (↑↑) and complete atrophy of fasciculi (↑↑) X 200.

Plate-3

T.S. of gastrocnemius muscle from denervated fenoterol treated mice at day 7, showing degenerated muscle fibers of variable sizes (↑), PMNL infiltrations around myofibers (↑↑) and myofibers are degenerating in almost whole muscle section X 400.

T.S. of gastrocnemius muscle from denervated fenoterol treated mice at day 14, demonstrating spaces between muscle fibers (↑), hypertrophied and variably shaped muscle cells (↑↑) and increased connective tissue between interfascicular spaces ( ) X 400.

T.S. of gastrocnemius muscle from denervated fenoterol treated mice at day 21, exhibiting merger and hypertrophy of muscle fibers (↑) and PMNL infiltration for scavenging atrophying muscle fibers (↑↑) X 400.

T.S. of gastrocnemius muscle from denervated fenoterol treated mice at day 28, showing merger and hypertrophy of muscle fibers (↑) and degeneration of atrophied muscle fibers (↑↑). Muscle fibers are returning towards normalcy along with subsarcolemmal nuclear disposition X 400.
Administration of fenoterol to denervated mice seems to have a marginal effect on distribution of connective tissue as characteristic to normal innervated muscle. Histopathological examination of denervated mice treated with fenoterol showed ameliorating effects in gastrocnemius which showed some recovery of muscle structure towards normal profile. Immediate effects of fenoterol treatment to denervated mice at day 7 were exhibited by slight degeneration in fibers and some PMNL infiltration around myofibers (Plate 3 A). Some of the fibers appeared hypertrophic amongst atrophying population. An extreme variability in fiber size amongst different population was observed. Connective tissue increased between interfascicular spaces associated with hypertrophied and variably shaped fibers at day 14 (Plate 3 B). Muscle fibers become merged and also showed hypertrophy at day 21. A very small population of cells, largely hypertrophic appeared healthy and showed cells which were largely round in their outline (Plate 3 C). PMNL infiltration decreased to a large extent showing recovery of muscle fibers from denervation stress after 28 days of fenoterol administration. Normal looking fibers with subsarcolemmal nuclei disposition were seen at this stage (Plate 3 D).

**DISCUSSION**

Chronic fenoterol administration for 28 days (1.4 mg/kg body weight) resulted in an increase in the body mass of mice. On day 28 of chronic treatment, weight of innervated fenoterol treated animals were significantly higher (32.7 ± 1.69 g) as compared to normal innervated control animals (29.5 ± 1.58 g), showing an increase of 10.85 % in body weight of treated mice over the control animals. There was an overall increase in gastrocnemius muscle weight to whole body weight (g) ratio after chronic treatment, from 7 to 28 days. This increase has been reported by some previous studies who elucidated that β-adrenoceptor agonists produce specific protein anabolic effects in skeletal muscle in addition to lipolysis in the adipose tissue of different vertebrates (Baker et al. 1984; Choo et al.1992; Kim et al.1991; Moore et al.1994; Kumar et al. 2003). The drugs are therefore employed in repartitioning and production of lean meat in commercial poultry and other animals (Elliot et al.1993). However, denervation resulted in decline in the ratio of gastrocnemius to body weight at all stages of study suggesting the onset of atrophy of constituent muscle fibres. It was further reported that the fenoterol treatment resulted in amelioration of muscle atrophy at all the stages studied and there was a significant increase of 4.91% at day 28 in denervated fenoterol treated animals. Similarly, dry muscle mass and total tissue protein of gastrocnemius muscle showed a progressive increase at all stages of chronic fenoterol treatment.

Denervation decreases total tissue protein, in part via increased protein degradation involving the lysosome, is supported by studies comparing distal and proximal denervation that suggested that the loss of a neurotrophic factor contributes to this mechanism of protein loss (Weinstein et al.1997). Denervation induced atrophy is ameliorated by administration of beta-agonists which is associated with increased protein kinase activity (Sneddon et al.2000). The present investigation depicted that treatment of denervated mice with fenoterol helped the muscles in reverting towards normal profile. Restoration of structural organization of muscle cells and nuclei almost characteristic to normal innervated muscle cells and changes in phenotypic expression of denervated muscle provide testimony to this. Present findings are supported by some earlier studies which revealed that the drugs can even reverse dystrophic states (Dupont-Versteegden et al.1995; Delday et al.1997). β-agonists can oppose muscle weaknesses in aged muscles (Agrawal et al. 2003; Carter et al. 1991; Zeman et al.1987). Cytoarchitecture of gastrocnemius muscle is organized with well defined populations of nuclei in the normal innervated animals (Katoch and Malhotra 1982; Garg and Sharma 2006a; Garg and Sharma 2006b and Katoch et al. 2006). Normal innervated mice with fenoterol administration results in muscle fibre hypertrophy, a common anabolic effects of β-agonist (Petrou et al. 1995). Polymorphonuclear leucocytes (PMNL) infiltration around severely damaged muscle fibers was a common feature. PMNL infiltration and macrophages are generally rich in the hydrolytic and other lytic enzymes indicating that the infiltration of these leucocytes as well as macrophages induces myodegeneration through lysosomal hydrolases and other proteolytic enzymes. Denervation of the muscle led to the onset of dedifferentiated state of the muscle tissue and a complete loss of an organized cellular architecture became noticeable. It is a well known fact that the biochemical as well as physiological effects of denervation becomes established in muscle tissue within a few days (Albuquerque et al.1972; Sharma and Malhotra 1994). Myonecrosis, as reported during present study, is characterized by peculiar anatomical changes in the nuclear morphology, change in the orientation as well as migration of the nuclei from their usual sarcolemmal positions to the centre of the fibers is an important pathological sign of muscle disease (Adams 1974; Sharma and Malhotra 1995). The most probable mechanism of this myonuclear loss is due to their apoptotic death (Rodrigues et al.1995; Dupont-Versteegden 2006). Putative apoptotic myonuclei in muscle tissue after denervation has also been reported (Borisov and Carlson 1995). Apoptosis in skeletal muscle and its relevance to atrophy has been revealed in detail (Dupont-Versteegden 2006). A number of disintegrating fibers were being phagocytised by the infiltrated PMNL and macrophages. The PMNL around these muscle fibers appeared extremely pycnotic and even demonstrated further splitting, a process analogous to apoptosis. It is difficult to ascertain whether these nuclei undergoing pycnosis belong to PMNL population or include muscle nuclei as well. One of the effects of fenoterol administration to denervated mice included a delay in the process leading to fiber atrophy. While atrophic cells remained common, other fibers with polygonal to circular outlines also made their appearance as early as within 7 days of denervation in the presence of fenoterol. It was associated with a decreased level of PMNL invasion of fibers, lesser degree of pycnosis in


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