REVERSAL OF HISTOPATHOLOGICAL CHANGES IN SOLEUS MUSCLE OF DENERVATED MICE BY FENOTEROL HYDROBROMIDE

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ABSTRACT
Amelioration of denervation stress by fenoterol hydrobromide has been investigated in soleus muscle of mice. Fenoterol hydrobromide is a β2-Adrenoceptor agonist which acts an anabolic agent in skeletal muscle, because they promote hypertrophy and have potential application for enhancing muscle repair after injury. A significant increase (>0.05) in ratio of soleus muscle to body weight was reported at every stage of study i.e. 7 to 28 days of chronic administration of fenoterol to normal innervated mice. This ratio in denervated animals showed a constant significant decline from 6.66% at day 7 to 54.54% at day 28 pointing towards stress induced by denervation in mice. Similarly, soleus muscle of denervated animals also showed a decrease in dry muscle mass. 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 soleus muscles which showed some recovery of muscle structure towards normal profile.

KEY WORDS: Mice, Soleus Muscle, Fenoterol Hydrobromide, Denervation

INTRODUCTION
Agents that stimulate an increase in muscle size (hypertrophy), by either increasing protein synthesis, decreasing protein degradation, or both, have the potential to be applied clinically to combat muscle wasting conditions (Lynch 2001, 2002). Pharmacological agents that promote muscle protein accretion have clinical potential for improving muscle regeneration after injury. Synthetic β2-adrenoceptor agonists (β2-agonists), such as clenbuterol and fenoterol, were initially developed for acute asthma treatment, to facilitate bronchial smooth muscle dilation (Van Nolte et al., 1974). Further investigations found that, when administered at higher doses, β2-agonists have an anabolic effect on skeletal muscle (Emery et al., 1984). Sympathomimetics such as β2-adrenoceptor agonists (β2-agonists) have therapeutic potential for conditions in which muscle wasting and weakness are indicated. These agents increase muscle size and strength of healthy muscle fibers and enhance the rate of muscle repair following injury (Beitzel et al., 2004; Emery et al., 1984; Ryall et al., 2002, 2004). The increase in muscle mass is due to β2-adrenoceptor mediated protein accretion via increase in intracellular cAMP promoting both an increase in protein synthesis and a decrease in degradation (Navegantes et al., 2002). These β-adrenergic agonists possess powerful skeletal muscle specific protein anabolic effects in addition to their lipolytic actions in different vertebrates (Choo et al., 1992; Kim and Sainz 1992; Kumar et al., 2003). Fenoterol treatment causes a small increase in fatigability due to a decrease in oxidative metabolism in skeletal muscle fibers (Larsson & Ramamurthy 2000; Rayll 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 (Pearen et al., 2009). Therefore, present study aimed at effects of β-adrenergic stimulation using fenoterol and its effects on structure and functions of skeletal muscles. Amelioration of stress induced damage in mice soleus 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. Soleus muscles were immediately excised and weighed. Soleus muscles were pooled together from four mice due to its small size.

ii) Dry muscle mass and total tissue proteins
Dry muscle mass of mice soleus 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 a progressive increase in the body weight of mice during chronic treatment of drug from 7 to 28 days. The increase in body weight was found to be 6.48% and 10.85% at 7 and 28 days respectively which was significantly higher (p<0.05) than the control mice. Fenoterol treatment to denervated mice showed 5.15% increase in body weight at day 7 and 3.80% at day 28 (Table 1; Figure 1).

TABLE 1 Effects of fenoterol hydrobromide (F) on body weight of mice from 7-28 days. Values are presented as mean ± SEM; *p<0.05 (n> 8). Mean weight of mice at the beginning was 25

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g) in Days</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>26.2±1.32</td>
<td>27.7±1.37</td>
<td>28.6±1.41</td>
<td>29.5±1.58</td>
<td></td>
</tr>
<tr>
<td>NF</td>
<td>27.9±1.51*</td>
<td>30.1±1.55*</td>
<td>31.2±1.59*</td>
<td>32.7±1.69*</td>
<td></td>
</tr>
<tr>
<td>% increase</td>
<td>6.48</td>
<td>8.66</td>
<td>9.09</td>
<td>10.85</td>
<td></td>
</tr>
<tr>
<td>Dn</td>
<td>25.2±1.31</td>
<td>26.3±1.35</td>
<td>27.6±1.39</td>
<td>28.9±1.51</td>
<td></td>
</tr>
<tr>
<td>DnF</td>
<td>26.5±1.49*</td>
<td>27.5±1.45*</td>
<td>28.3±1.51</td>
<td>30.0±1.55</td>
<td></td>
</tr>
<tr>
<td>% increase</td>
<td>5.15</td>
<td>4.56</td>
<td>2.54</td>
<td>3.80</td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 1: Effects of fenoterol hydrobromide (F) on body weight of mice from 7-28 days. Values are presented as mean ± SEM; *p<0.05 (n> 8). Mean weight of mice at the beginning was 25

N-normal; F-fenoterol; Dn-denervated; DnF-denervated+fenoterol

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Increase in soleus muscle weight to whole body weight at day 28 was recorded as 36.36% in fenoterol treated normal innervated animals. The soleus to body weight ratio in denervated animals showed a constant significant decline from day 7 to day 28, which showed a little improvement after chronic administration of fenoterol (Table 2; Figure 2). Dry soleus mass in normal innervated mice was 216.7 ± 2.80 µg/mg of fresh tissue weight at day 28 of the study. It demonstrated an increase of 8.07% (234.2 ± 2.16) in innervated treated mice at the corresponding day of study. Denervated animals showed a decrease in dry soleus mass. It decreased to 3.50% in denervated group as compared to normal innervated mice on day 7 of the study and reached to a significant decreased level of 27.41% at day 28 of chronic treatment. The effects of fenoterol on denervated mice healed the effects of denervation but its value was significantly below the normal innervated levels. Moreover, there was a constant increase in effectiveness of the drug with increase in number of days of administration up to day 28 of the study (Table 3; Figure 3).

**TABLE 2:** Changes in soleus 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>
<thead>
<tr>
<th>Groups</th>
<th>Soleus/body weight ratio (%) in Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>N</td>
<td>0.15±0.003</td>
</tr>
<tr>
<td>NF</td>
<td>0.18±0.002*</td>
</tr>
<tr>
<td>% increase</td>
<td>20.00</td>
</tr>
<tr>
<td>Dn</td>
<td>0.14±0.004*</td>
</tr>
<tr>
<td>DnF</td>
<td>0.15±0.002*</td>
</tr>
<tr>
<td>% increase</td>
<td>7.14</td>
</tr>
</tbody>
</table>

**FIGURE 2:** Changes in soleus 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 soleus (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).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dry muscle mass (µg/mg fresh tissue weight) in Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>N</td>
<td>185.6±0.78</td>
</tr>
<tr>
<td>NF</td>
<td>190.8±0.67</td>
</tr>
<tr>
<td>% increase</td>
<td>2.80</td>
</tr>
<tr>
<td>Dn</td>
<td>179.1±1.19*</td>
</tr>
<tr>
<td>DnF</td>
<td>185.9±0.56*</td>
</tr>
<tr>
<td>% increase</td>
<td>3.79</td>
</tr>
</tbody>
</table>
Total tissue proteins in soleus muscle showed an increasing trend after daily fenoterol treatment. 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 12.53% at day 28 (Table 4; Figure 4).

**TABLE 4:** Changes in total tissue proteins of soleus 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).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total tissue proteins (µg/mg fresh tissue weight) in Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>N</td>
<td>179.3±0.95</td>
</tr>
<tr>
<td>NF</td>
<td>184.4±1.22</td>
</tr>
<tr>
<td>% increase</td>
<td>2.84</td>
</tr>
<tr>
<td>Dn</td>
<td>173.6±0.91</td>
</tr>
<tr>
<td>DnF</td>
<td>176.2±0.94</td>
</tr>
<tr>
<td>% increase</td>
<td>1.49</td>
</tr>
</tbody>
</table>

**FIGURE 4:** Changes in total tissue proteins of soleus 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).
ii) Histopathological examination

Soleus muscle

Soleus muscle in normal innervated mice exhibited polygonal, circular or oval shaped fibers with normal fasciculi and nuclei aligned along subsarcoleminal region (Plate 1 A, B). Treatment of normal innervated mice with fenoterol induced hypertrophy and leucocyte infiltration around epimysial regions at day 7 (Plate 1 C). Interfibrillar spaces increased along with hypertrophy and degeneration of muscle fibers at days 14 and 21 (Plate 1 D, E). Interfibrillar spaces were clearly noticed. Muscle hypertrophy was highly associated with heavy leucocyte infiltration showing degeneration of fibers at day 28. Merged fibers were frequently seen in the muscle sections (Plate 1 F).

Plate 2

A, B: T.S. of soleus muscle from denervated mice at day 7, revealing some atrophied fibers (↑) and PMNL infiltrations around myofibers (↑↑). Muscle fibers are sparsely placed (B) X 400.

C: T.S. of soleus muscle from denervated mice at day 14, exhibiting merging of fibers (↑), hypertrophied fibers (↑↑) and pycnotic nuclei (AX) X 400.

D: T.S. of soleus muscle from denervated mice at day 21, depicting complete myofibrillar degeneration of muscle fibers (↑) and fibers merging (↑↑) X 200.

E, F: T.S. of soleus muscle from denervated mice at day 28, demonstrating collagen infiltrations along outer margin (↑) and degeneration of fibers (↑↑) X 200 (E), X 400 (F).

Deneravation induced stress due to lack of neurotrophic regulation resulted in atrophy of fibers accompanied by leucocyte infiltration. Normal polygonal shape or outline of the muscle was lost and they appeared disfigured. Atrophied muscle fibers formed triangular bundles showing reduction in size of fibers (Plate 2 A, B).
Histopathological changes in soleus muscle by fenoterol hydrobromide

Individual fibers started merging with each other at day 14 of denervation. Nuclei of individual muscle fibers encircled by PMNL had become pycnotic (Plate 2 C). Atrophy, disfiguration and degeneration of fibers associated with collagen infiltration was observed at day 21 and 28. Muscle sections revealed cells densely surrounded by nuclear aggregates around the sarcolemma. Massive infiltration of PMNL and other macrophages was clearly visible. Broadening of interfibrillar spaces were clearly marked and some cells showed sign of connective tissue formation (Plate 2 D, E, F). However, treatment of denervated mice with chronic doses of fenoterol pointed towards ameliorating effects of drug. At day 7, only some hypertrophied fibers with some PMNL infiltration were reported in denervated treated mice (Plate 3 A). Effects of fenoterol treatment were exhibited by modification of muscle cell, nuclear structures and accumulation of collagen. Muscle fibers demonstrated clear outline with subsarcolemmal placed nuclei. Some infiltration of PMNL around muscle fibers was observed. Some fibers were normal in shape with single nucleus on the periphery. Clumps of nuclei were observed near the merged fibers (Plate 3 B-F).

**PLATE 3**

A: T.S. of soleus muscle from denervated fenoterol treated mice at day 7, showing muscle hypertrophy (↑) and PMNL infiltrations around myofibers (↑↑) X 200.
B: T.S. of soleus muscle from denervated fenoterol treated mice at day 14, showing muscle hypertrophy and degeneration (↑). Muscle fibers are totally devoid of nuclei X 400.
C, D: T.S. of soleus muscle from denervated fenoterol treated mice at day 21, revealing fibers merging, elongation, hypertrophy (↑) and variably shaped nuclei (↑↑) X 200 (C), X 400 (D).
E, F: T.S. of soleus muscle from denervated fenoterol treated mice at day 28, depicting atrophied fibers (↑) and extensive collagen infiltration along the margins (↑↑) X 200 (E), X 400 (F).

**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.

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 with in 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 muscle nuclei and less extensive myonecrosis in denervated mice fed with fenoterol. Histopathological data further revealed that as the post-denervation period increases, the reversal of fiber atrophy emerges more and more conspicuous. Therefore, chronic administration of fenoterol resulted in a differential recovery process.

The utility of beta adrenergic agonists in ameliorating atrophic state lies in their ability to promote biosynthesis and thereby result in fiber hypertrophy. This is well supported by an increased mRNA synthesis and protein levels after the administration of beta agonists to denervated animals (Albuquerque et al. 1972; Sharma & Malhotra 1994; Adams 1974; Sharma & Malhotra 1995). The fiber dimensions kept on registering a continuous increase towards 28 day post-denervation especially as a result of fenoterol administration, does lend credence to the view that beta agonists do promote growth (Rodrigues & Schmalbruch 1995; Dupont-Versteegden 2006). Muscle cells tend to return towards normalcy due to the effects of beta-agonists (Borisov & Carlson 1995) and denervation atrophy in rat soleus muscle if ameliorated by beta-agonists (Sneddon et al. 2000).

Soleus muscle in normal innervated mice exhibited polygonal, circular or oval shaped fibers with normal fasciculi and nuclei aligned along subsarcolemmal region. Treatment of normal innervated mice with fenoterol induced hypertrophy and leucocyte infiltration around epimysial regions. Muscle hypertrophy with PMNL infiltration showing degeneration of fibers reached its highest level on day 28. However, denervation atrophy, a typical pathological state is characterized by muscle wasting which results from the breakdown of cytocontractile apparatus, mainly myofibrillar proteins, as a result of stepped up proteolysis (Maltin et al. 1986, 1989; Helferich et al. 1990). Chronic administration of fenoterol to denervated mice exhibited a reduction in degeneration to some extent which is in agreement with the findings of Garg and Sharma (2006 a, b). Muscle fibers of fenoterol treated mice showed majority of hypertrophied fibers with long elongated fibers, which thereby disturbed the fiber heterogeneity as observed in normal muscle fibers. Some studies have documented neuroprotective action of fenoterol by induction of growth factors after cellular damage (Grant et al. 1993). 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 and Maltin 1997). β-agonists can oppose muscle weaknesses in aged muscles (Agrawal et al. 2003; Carter et al. 1991; Zeman et al. 1987).

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