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Clenbuterol Attenuates Work Stress Induced Degeneration in Rat Skeletal Muscle and Its Inhibition by Butoxamine

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Abstract : Clenbuterol is known to have therapeutic potential in ameliorating muscle atrophy because of its presumed anabolic effects. However, little is known about its effects on lipids under normal and stress conditions. Male rats of Wistar strain were shedied under work stress and clenbuterol treatment to understand the lipid status. Animals were subjected to work over load stress and then treated with clenbuterol (2 mg/kg/day) to find out its potential in recovery from stress. β_2 -antagonist butoxamine (2 mg/kg/day) was also given to another clenbuterol treated group to study its β blocking efficacy. Work stress decreased lipid levels in skeletal muscles, which is reflected by lowering cholesterol and triglyceride levels in the skeletal muscles whereas an increase in the two lipid fractions has been observed with clenbuterol which also induced skeletal muscle hypertrophy in normal animals and attenuated degenerative changes in skeletal muscles under work stress. Increased lipids in the heart by clenbuterol infer towards its deleterious effects on heart. Antagonist butoxamine had stimulatory effects similar to clenbuterol initially where an increase in the lipid levels was observed, which however were reduced during successive stages indicating its inhibitory effect later on. Butoxamine also prevented muscle hypertrophy which was brought about by clenbuterol, without affecting degenerative changes induced by work stress.

Key Words : Clenbuterol, Butoxamine, β-Adrenoceptors, Lipids, Skeletal muscles

Introduction :

Clenbuterol is a direct acting β sympathomimetic agent that is known to be anabolic and believed to impart muscle gain (Choo *et al*., 1992; Kim *et al*., 1992; Maltin *et al*., 1992; Lynch *et al*., 1996) that has been attributed to accelerated protein turnover rate (Horne and Hesketh, 1990). The only medicinal use for which clenbuterol is generally prescribed is for the treatment of obstructed airways. It interacts directly with β adrenoceptors with or without sympathetic activity in a dose dependant manner (Haycock, 1998) and is known to attenuate skeletal muscle atrophy (Maltin *et al*., 1987; Carter *et al*., 1991; Dupont *et al*., 1996; Sneddon *et al*., 2000; Zeman *et al*., 2000; Aggarwal *et al*., 2003). Clenbuterol has shown a neuroprotective action in the central nervous system by induction of growth factors after cellular damage (Frerichs *et al*., 2002). In addition to accelerated protein turnover rate β adrenoceptor agonists are also known to increase markedly the catabolic and decrease the anabolic lipid metabolic processes consequently leading to decreased fat deposition (Mersmann, 2002, Belahsen and Deshais, 1992).

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Skeletal muscle is particularly vulnerable to shortfalls in the production of mitochondrial ATP because of the high metabolic demands of muscle work and lipids are an important source of metabolic energy for sustained work in skeletal muscles. During heavy exercise, a dramatic increase occurs in the fat utilization which results entirely from release of epinephrine and norepinephrine (Guyton, 2000). Reduction of exercise induced degeneration and slow to fast fibre transformation in skeletal muscles by clenbuterol is also well known (Zeman *et al*., 1988). In addition to skeletal muscle mass gains and reversal of muscle atrophy, clenbuterol also induces cardiac hypertrophy (Duncan *et al*., 2000) and is also known to have myotoxic effects on heart and soleus muscle (Burniston *et al*., 2002).

β adrenergic blocking agents on the other hand selectively and competitively block the action of catecholamines as well as β agonist (synthetic analogs of catecholamines), mediated through β adrenoceptors thereby reducing the stimulatory effects of the sympathetic nervous system as elicited by β adrenergic agonists. Butoxamine is a selective β_2 antagonist which blocks the vasodilator and metabolic effects of β receptor stimulation. The antagonists improve exercise tolerance in patients with angina and inhibit adrenaline-induced glycogenolysis in the skeletal muscles as well as release of free fatty acids from adipose tissue. Butoxamine has also been found to revert slow to fast fibre transformation in skeletal muscles of rat as induced by clenbuterol (Zeman *et al*., 1988) and inhibit the fat cell enzyme stimulated by β agonist, isoproterenol (Kather and Simon, 1980).

In view of the available data it was thought worthwhile (a) to see the effect of work overload stress on the skeletal muscles and heart of rats, (b) to find out the effect of clenbuterol on normal as well as stressed animals and (c) to know whether β blockade by butoxamine is able to inhibit clenbuterol induced effects.

Materials and Methods :

Adult male Wistar rats weighing (120-150g) were obtained from Central Research Institute (CRI), Kasauli, India and maintained in the animal house of the department under suitable hygienic conditions i.e. light (16 h daylight) and temperature (24 ± 2^0C). Animals were provided standard pellet diet (Hindustan Lever Ltd.) and water *ad libitum*. Clenbuterol hydrochloride and butoxamine hydrochloride were obtained from Sigma Chemical Co., USA and all the other chemicals used were of highest purity and analytical grade.

Rats were divided into six groups. Group I (normal) served as control. Group II rats were denervated (sciatic nerve of left hind limb was cut ~1 cm) as per the method of Dhingra *et al* (1978). Denervation resulted in hind limb paralysis as a result of which the contralateral limb was subjected to continuous workoverload. Because of imbalance created in its movements due to paralyzed hind limb the *pectoralis* muscle was also subjected to certain amount of work stress. Group III included rats, given clenbuterol daily intraperitoneally (2 mg/kg body wt. for 15days). Group IV animals were also given clenbuterol similar to group III but also received butoxamine

hydrochloride (2 mg/kg body wt. for 15 days). Group V and VI had denervated rats that received similar treatments as group III and IV respectively. The rationale for dose of drug given was arrived at on the basis of previous studies, according to which 1-2 mg/kg/day dose of β-agonist was effective in inducing muscle hypertrophy (Carter *et al* 1991).

All the animals were maintained under similar experimental conditions for a period of 30 days and were sacrificed on day 7 and 30 of post-denervation by cervical dislocation. Atleast 4-6 animals from each group were sacrificed at each stage. *Gastrocnemius* from the contralateral limb, *pectoralis* and heart were excised immediately and processed for biochemical, histological and histochemical studies. Bouin's fixed tissues were used for histology whereas tissues to be used for histochemistry were stored at 4^0 C till further use and the tissues for biochemical study were immediately employed for lipid extraction (Folch *et al* 1957). Lipid extract formed was estimate cholesterol quantitatively (Stadman 1957) using sulphuric acid and acetic anhydride. Similarly triglycerides were estimated by the method of Vanhandel & Zilversmith (1957) using arsenic trioxide and chromotropic acid. Total lipids were histochemically localized in the cryostat cut thin sections (7μ) of *gastrocnemius* and *pectoralis* as per Baker (1946) using Sudan Black 'B' whereas haematoxylin–eosin staining helped to see the histopathological changes.

Statistical significance was determined by student's *t*-test (Pearson and Hartley, 1960) to find out significance of main differences among the groups. Differences were assumed significant at $P<0.01$ and $P<0.001$.

Result and Discussion :

Cholesterol and triglyceride levels in *gastrocnemius*, *pectoralis* and heart of control as well as experimental group of animals are presented in Table 1. Animals subjected to workoverload show significantly decreased cholesterol levels in all the muscles under study, except the heart where the decrease is non-significant. In contrast to the study of Crouse *et al*. (1972) where the cholesterol content increases with age, decreased cholesterol levels are observed on day 30 when compared to day 7 during the present study. It is known that with intensive exercise, glycogen phosophorylase is downregulated if fatty acids are available (Dyck *et al.*, 1996). The muscles under work stress for energy production utilize lipids, as they are the richest source of energy. Clenbuterol is known to possess lipolytic capabilities (Choo *et al*., 1992; Emery *et al*., 1984) but on the contrary during the present study significant increase in the two lipid fractions is observed with clenbuterol administration. Since β receptors are present on the adipose tissue hence the drug probably binds to these receptors thereby activating the hormone sensitive lipase, which in turn stimulates the lipolysis (Haycock, 1998). The lipids hence lipolysed are mobilized to the other tissues and particularly to the muscles where they are much needed (Mersmann, 2002; Sharma and Garg, 2003).

Drug administration to the animals subjected to workoverload increased the lipid levels to near normal and above normal. Increase in the cholesterol content of all the three muscles with clenbuterol is highly significant. Similarly significant increase

Table - 1 : Effect of butoxamine (2 mg kg^{-1} day^{-1}) and clenbuterol (2 mg kg^{-1} day^{-1}) on cholesterol and triglyceride levels [mg/g fresh tissue wt.]of *gastrocnemius, pectoralis* and heart of rats under work overload stress.

[Values are mean ±SE from 6 observations in each group]

	Gastrocnemius				*Pectoralis*				Heart			
Groups	Cholesterol		Triglycerides		Cholesterol		Triglycerides		Cholesterol		Triglycerides	
	7	30	7	30	7	30	7	30	7	30	7	30
I [N]	6.133± 0.230	2.578± 0.153	177.91± 3.118	356.17± 29.773	4.56± 0.189	1.973± 0.083	211.99± 4.758	66.44± 17.945	0.717± 0.064	0.706± 0.110	75.21± 3.222	75.44± 6.014
II [W]	2.47± 0.008**	1.514± 0.038**	259.19± 47.896	64.95± 0.085**	2.76± 0.051**	1.538± 0.032**	164.52± 29.414	74.94± 9.616	0.656± 0.033	0.561± 0.094	299.41± 16.171**	103.66± 9.991
III [NC]	7.234± 0.034*	2.986± 0.101	195.28± 1.236**	379.92± 2.568	4.979± 0.112	2.522± 0.048**	311.45± 1.388**	136.35± 2.306*	0.947± 0.028*	0.925± 0.056	76.34± 1.262	76.92± 2.346
IV [NCB]	8.007± 0.529	3.505± 0.074**	183.59± 2.486**	365.75± 38.133	5.490± 0.540	2.715± 0.083	366.83± 5.153**	168.173± 4.888**	1.132± 0.284	1.176± 0.118	69.167± 2.217*	50.98± 3.068**
V [WC]	3.46± 0.044**	1.982± 0.025**	233.54± 43.042	150.59± 11.710**	4.79± 0.035**	2.221± 0.003**	294.98± 11.380**	192.00± 2.984**	1.256± 0.069**	1.198± 0.369	377.38± 43.986	124.88± 31.270
VI [WCB]	4.074± 0.330	2.170± 0.082	344.81± 4.598	85.510± 3.184**	3.732± 0.338*	2.340± 0.094	308.68± 4.407	145.30± 3.499**	1.580± 0.228	1.086± 0.124	316.52± 6.44	86.81± 3.596

P value: * < 0.01; ** < 0.001

N= normal; W= work overload; C= clenbuterol; B= butoxamine

in the triglyceride content of *gastrocnemius* and *pectoralis* was observed which however was non- significant in the heart. Return of lipid levels which are reduced under work stress towards normalcy by clenbuterol suggest that it is helpful in recovery from stress but at the same time increase in the heart lipids indicate its negative influence as well (Wexler and Greenberg, 1978; Vijayapadma and Shyamala Devi, 2002). Butoxamine supplementation to clenbuterol treated animals does not seem to induce any appreciable differences; instead a further increase in the lipid levels is observed initially; This suggests that the antagonist while binding to the receptor site has a stimulatory effect initially instead of immediate and complete β blockade. However significantly decreased triglyceride levels observed in *gastrocnemius* and heart on day 30, indicates that butoxamine, is able to inhibit clenbuterol-induced effects much later. Similarly, the animals under work stress also show increase in the cholesterol content when treated with clenbuterol and butoxamine. However a decrease in the triglyceride levels of all the muscles is noticed, which is in confirmation with the studies of (Kather and Simon, 1980) where the fat cell enzyme stimulated by β agonist was inhibited by butoxamine. It is also known that cardiac response to exercise and other situation, in which sympathetic tone is increased, is attenuated by the antagonists.

Studies on the physiology of a large number of tissues under various stress conditions have shown to change the lipid profile (Sharma and Malhotra, 1991). Histochemical localization of total lipids in the *gastrocnemius* and *pectoralis* muscles reveal heterogeneous lipid distribution pattern, on the basis of which three different fibre types are distinguishable (Brooke and Kaiser, 1970). There are three types of muscle fibres called white, intermediate and red differing in ultrastructure, activities of oxidative and glycolytic enzymes, myoglobin content and certain properties of myosin (Talesara and Kiran, 1986). During normal growth, the skeletal muscle fibres differentiate into fibre types in which lipid-rich and lipid-poor muscle fibres are segregated from the basic stock of homogenous lipid rich fibres (Cosmos, 1970). Normal muscles show type I (slow twitch, oxidative) fibres taking up maximum stain whereas type II B (fast glycolytic) fibres are least stained and the third type II A stained moderately, expressing the amount of lipids present in them (Fig. 1a). Under the conditions of workoverload both the muscles exhibit almost identical response with respect to sudan staining (Fig. 1 b and c). Lesser lipids are seen in all the three types of fibres, which is in close conformity with the biochemical results. Slow fibres are involved in more prolonged movements and are stimulated at continuous low frequencies. Therefore they contain maximum lipids under normal conditions which are rapidly utilized under stress (Guyton, 2000, Sharma and Malhotra, 1993). During the later stages, exercise leads to hypertrophy of muscle fibres as a result of which lipids probably are used in the formation of new membrane systems (Sharma and Malhotra, 1993), as lipids constitute an integral part of the cell membranes. Clenbuterol treated normal animals show hypertrophied fibres as well as extensive lipid accumulation in nearly all the three types of fibres (Fig. 1d) which disturbs the fibre heterogeneity achieved on the basis of Sudan staining. On the other hand β-agonist treatment to the stressed animals brought the lipid distribution pattern back towards normalcy (Fig. 1e) thereby indicating therapeutic potential of the drug in recovery from stress. Lesser lipid content is

noticed in butoxamine supplemented, clenbuterol treated animals (Fig. 1f), which suggests β blockade by the antagonist that prevents clenbuterol expression.

During the present study, histology is intended to understand the structure of normal muscle and histopathological aberrations induced under stress conditions. Morphologically the fibres are distinguished on the basis of their cross sectional dimensions, number of myofibrils and sarcoplasmic granulation. *Gastrocnemius*

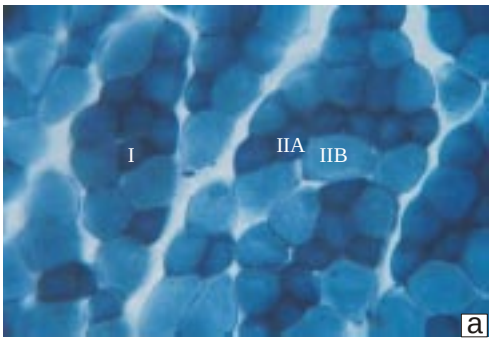

Fig 1 : Histochemical localization of total lipids showing fibre heterogeneity where three main types of fibres (I, IIA, IIB) are distinguishable in *gastrocnemius* of (a) normal (b&c) exercised *gastrocnemius* and *pectoralis* at day 7 and 30 respectively showing less lipids, (d) increased lipid content is seen with clenbuterol treatment on day 30 in normal *gastrocnemius* and (e) recovery in lipids is seen in exercised *pectoralis* on day 7 (f) butoxamine and clenbuterol treated normal *gastrocnemius* on day 30 showing nearly normal lipid distribution pattern. (SBB× 200).

and *pectoralis* muscles show round to oval shaped fibres with subsarcolemmal position of nuclei under normal conditions (Fig. 2a) in contrast to the skeletal muscles of animals under work stress where hypertrophied muscle fibres can be seen as early as on day 7 (Fig. 2b). According to Sharma and Malhotra (1995), anabolic processes are stimulated as a result of continuous exercise. Gutmann (1962) holds a view that compensatory hypertrophy of contralateral muscle is evoked by increased fibral activity of the muscles on this side that therefore results in muscle hypertrophy. Nuclear component in normal skeletal muscles comprise of intrafibril and interfibril nuclei, subsarcolemmaly positioned. Exercised muscles show the presence of activated intrafibrillar nuclei that become highly vesicular and an explanation for stimulated muscle fibre growth. Interfibrillar nuclear proliferation is also observed on day 7 as well as on day 30 where clusters of nuclei with variable shapes are seen in the interfibrillar spaces (Fig. 2c), which thereby alter the metabolism of those fibres and subsequently result in degeneration later. Displacement of nuclei from their normal subsarcolemmal position is the most common abnormality involving muscle when under stress (Karpati, 2001). During the later stages *i.e.* on day 30 muscle fibres undergo pathological changes like sarcolemmal breakdown, myofibrillar degeneration in the form of pinhead foci, fibre shape changes and sarcoplasmic necrosis (Fig. 2d). Continuous exercise probably nullify the regulatory effect of the neurotrophic factors resulting in the induction of pathological conditions when the tissue itself does not get any time to effect repairs in the damaged components (Sharma and Malhotra, 1995).

Muscle fibres of clenbuterol treated animals show majority of hypertrophied fibres with long elongated fibres, which thereby disturbs the fibre heterogeneity as observed in normal muscle fibres. Amount of degeneration is lesser than non-treated animals on day 30, which indicates its contribution in attenuating muscle loss. Also a certain amount of proliferated non-contractile element (connective tissue) is also observed (Fig. 2e). Clenbuterol is known to induce muscle hypertrophy by increasing proteosynthetic processes (Hesketh *et al*., 1992) and regulating proteolysis (Navegantes *et al*., 2002). Group VI animals (stressed with butoxamine and clenbuterol) show nearly similar pattern of muscle degeneration as seen in stressed animals on day 30 where degenerating muscle fibres with variably shaped nuclei are prominent (Fig. 2f). This suggests that butoxamine blocks the β adrenoceptor site and hence prevents clenbuterol from binding to latter thereby leading to no response. This is in congruence with the studies of (Zeman *et al*., 1988) who was able to see reversal of slow to fast fibre transformation as induced by clenbuterol, with butoxamine.

Keeping the above in view, it could be safely concluded that workoverload stress leads to muscle hypertrophy initially but progresses towards muscle degeneration later on. Lipids present in the skeletal muscles are also utilized to meet increased energy demands as well as for laying down new membrane system and muscle repair under such a stress. β agonist clenbuterol though attenuates work stress induced degenerative changes but at the same time raised lipid levels in heart and connective tissue proliferation in skeletal muscles; this points towards its negative effects too. Butoxamine

supplementation to clenbuterol treated animals however suppresses some of the negative effects like increased heart lipids to an extent, but at the same time also inhibits clenbuterol from preventing muscle injury by workoverload. This keeps us in a state of dilemma to point on clinical importance of both the drugs in muscle wasting diseases.

Acknowledgement :

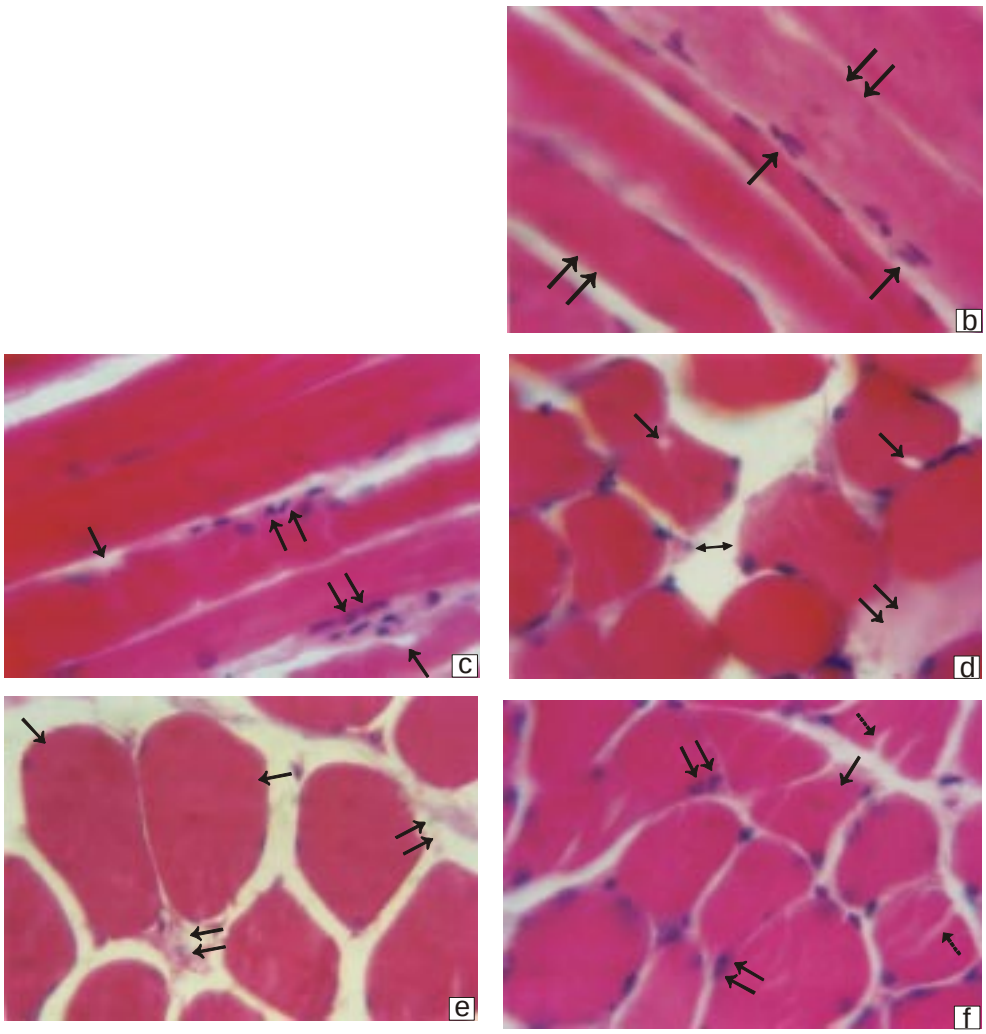

Fig 2 : Haematoxylin –eosin stained (a) normal *gastrocnemius* showing round fibres with subsarcolemmal position of nuclei (↗), (b) L.S. of exercised *gastrocnemius* demonstrating hypertrophied fibres (↗↗) with nuclear chains (↗) on day 7, (c) exercised *gastrocnemius* (L.S.) showing fibres with sarcolemmal breakdown(↗), variably shaped nuclear clustering (↗↗) on day 30 (d) (T.S.) of exercised *pectoralis* on day 30 showing fibre degeneration (↔), pin head foci (↗) and proliferated connective tissue (↗↗), (e) *pectoralis* muscle treated with clenbuterol show hypertrophied fibres (↗) as well as slight amount of connective tissue (↗↗) on day 7 (f) clenbuterol and butoxamine treatment demonstrate fibres exhibiting variable fibre shapes (↗) which are fragmented at places (⇢) and variably shaped nuclei are visible (↗↗). (HE × 900)

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