ABSTRACT

Clenbuterol is famous for having protein anabolic and lipolytic actions but recently it has been related to malfunctioning of heart. The present study was designed to investigate the deleterious effects of an acute dose (20 mg/kg body weight) of β-agonist clenbuterol on mice ventricular tissue. This was achieved by performing histology, lactate dehydrogenase enzyme assays and electron microscopy. The studies were carried out after 4, 8, 20 and 48 hours after drug administration to mice. The enzyme lactate dehydrogenase exists in five isozymic forms. LDH₁ and LDH₂, are the maker isoenzymes for studying cardiac injury as their serum analysis provides evidence of suspected tissue damage. From microscopic observations it was deduced that the fiber degeneration, nuclear pycnosis, mitochondrial edema and cristal disruption, were the key deviations in the cardiac muscle post drug administration. Ventricular lactate dehydrogenase enzyme demonstrated escalation in its levels after 4, 8 and 20 hours and a reduction was witnessed only at 48 hour stage. Heart specific isoform LDH₁ and LDH₂ also displayed a similar pattern. It was assumed that besides structural deviations, a single acute dose of 20 mg/kg body weight of clenbuterol caused alterations in the energy requirements in the ventricular tissue in mice. This was supported by extensive rise in the LDH levels at initial stages of the experiment and when the myocytes were not capable to bear the drug induced stress they got disintegrated resulting in release of the LDH in to serum.

KEYWORDS: Clenbuterol, lactate dehydrogenase, heart, serum, isozymes.
INTRODUCTION

β₂-Agonists are on the list of prohibited substances in sports. Clenbuterol has recently gained popularity as the slimming drug of choice due to its ability to reduce fat quickly. The anabolic and repartitioning effects of β-AR agonist clenbuterol have been widely investigated principally at doses ranging from 1-5 mg/kg in a variety of sedentary laboratory and live stock animals.¹ ² More recently the combined effects of clenbuterol administration in the above mentioned doses and exercise have been found to show a decrease in exercise performance³ ⁴ and a high incidences of sudden cardiac failure.⁵ High doses and frequent, prolonged administration may be mostly associated with possible adverse β-agonist action. The present study was therefore designed to investigate the deleterious effects of an acute dose of β-agonist clenbuterol on mice ventricles.

MATERIALS AND METHODS

Animals

Adult male Balb/c Mice (20-25 g) procured from Central Research Institute (CRI), Kasuali (H.P.), India, were maintained in the animal house of the Department of Biosciences of Himachal Pradesh University, under suitable hygienic conditions. The mice were provided feed and water ad libitum. All procedures adopted in animal care and methodologies, had the approval from Institutional Animal Ethics Committee of the University (Registration No. IAEC/Bio/7/2004).

Experimental Groups

Normal healthy animals were divided into two groups. Mice in first group served as control animals. Animals in second group were treated with clenbuterol.

Drug Administration

Clenbuterol was purchased from Sigma Aldrich USA. Stock solution of the β-agonist was prepared. Each mouse received a single dose of clenbuterol (20 mg/kg b. w.). The control animals were given an equal volume of vehicle i.e. saline. Mice were sacrificed 4, 8, 20 and 48 hr after the administration of β-agonist clenbuterol.

Histological Studies

Haematoxylin-Eosin staining.
**Biochemical Analysis**

**Sample Preparation and Separation of Serum**

The ventricles were homogenised in 0.2M Tris HCl buffer, (pH 7.3), for biochemical studies. Homogenate was centrifuged at 4000 rpm. Serum samples were prepared by keeping the blood as such overnight at 8 °C. Blood cells coagulated and serum was separated as a distinct layer. Protein concentration was determined by the method of Lowry et al.[6]

**Quantitative Determination of LDH**

Total LDH activity of the serum as well as all ventricular tissue samples was determined by employing a standard LDH assay.[7] Reaction velocity was determined by a decrease in absorbance at 340 nm, following an oxidation of NADH. Change in absorbance was finally converted into units of LDH activity. One unit of LDH activity was considered equivalent to an oxidation of one micromole of NADH per minute at 25 °C and pH 7.3 under the specified conditions. Reaction mixture for LDH activity contained, 0.2 M Tris (pH 7.3), 6.6 mM NADH and 30 mM sodium pyruvate.

**Native Polyacrylamide Gel Electrophoresis (PAGE)**

Mice ventricles were homogenised in 0.9% saline to get a 10% homogenate. Different homogenate was centrifuged at 4000 rpm for 30 minutes at 4 °C. Serum was prepared and LDH isozymes were resolved on a 7.5% gel, employing native polyacrylamide gel electrophoresis (PAGE), according to the method of Cooper.[8]

Tris HCl was used as buffer for separating (0.5 M; pH 8.8) as well as stacking (1.5 M; pH 6.8) gel. Tris glycine buffer (pH 8.3) was used as a running buffer. LDH isozymes were finally visualized by incubating the gels in a medium containing, 100 mM Tris (pH 7.0), 0.4 mg/ml Nitro BT, 0.7 mg/ml NAD, 0.5 mg/ml phenozine methosulphate (PMS) and 100 mM sodium lactate (pH 7.0), for 20 minutes at 37 °C. Different LDH isozymes appeared as dark (blue/violet) bands. Densitometry was also performed to evaluate the altered isozymic expression after drug administration.

**Electron Microscopy**

Transmission electron microscopy (TEM) was performed at All India Institute of Medical Sciences (AIIMS), Delhi, India. Mice ventricular tissue was carefully excised and fixed in fixative for 12 h at 4 °C. The fixed tissues were then washed four times in 0.2 M phosphate buffer and postfixed in 1% osmium tetroxide for 2 hours. They were dehydrated in graded
acetone steps and embedded in araldite CY212 and stained with uranyl acetate and lead citrate.

Statistical Analysis
Data was expressed as mean ± S.E.M. The statistical significance was determined by the application of Student t-test and one way ANOVA to find the mean difference between the groups. Post hoc (Dunnett and Turkey), tests were also performed. The differences were assumed significant at $P<0.05^*$.

RESULTS
Histological Observations
Cardiac muscle consisted of long anastomosing network of muscle fibers with randomly distributed nuclei in control mice ventricles (Fig. 1A). The nuclei of the cardiac myocytes lied in the middle of the cells. Each muscle fiber was surrounded by an endomysium of delicate connective tissue with a rich capillary network. Although the reticular fibers of the endomysium were not usually seen, one can see nuclei of fibroblasts between the muscle fibers and also many capillaries running alongside them. Fibroblast nuclei tend to be more flattened and darkly stained than those of cardiac muscle cells and were of course peripherally located.

The nuclei of cardiac fibers were near the middle of the cross section. Connective tissue ran between bundles of myocytes. Many capillaries could be seen within the connective tissue or at the periphery of a muscle fiber. The outer covering of the heart was represented by epicardium.

After 4 hr of clenbuterol treatment, the myocardial tissue still maintained the anastomosing network of muscle fibers. Myocyte nuclei were seen in the centre though some fibers illustrated loss of nuclei. This enucleation was fairly visible in the centre of the section (Fig. 1B). There were some very lightly stained areas with fibers undergoing myonecrosis. Enucleation of muscle fibers multiplied with the passage of drug administration time. After 8 hr, the myocardial nuclei were difficult to figure out in most of the fibers which were undergoing degeneration (Fig. 1C). There was significant myonecrosis after 20 and 48 hr of clenbuterol administration. Entire population of nuclei had turned pycnotic. There was large assembly of polymorphonuclear lymphocytes (Fig. 1D). There was necrotic fiber on one side
whereas a quite normal fiber was also visible. Macrophages nuclei were making long chains around the necrotic fiber.

**Lactate Dehydrogenase**

**Ventricle (Fig. 2)**

Control mice heart ventricles showed a total LDH activity of 0.386±0.058 U/mg protein. Clenbuterol administration first resulted in enhanced expression of the LDH concentration i.e. after 4 hr the activity was 0.425±0.038 (↑10%) and similarly at 8hr, 0.463±0.037 (↑20%) and 20 hr 0.466±0.027 (↑21%). It is only after 48 hr, that the LDH activity showed decline and became 0.213±0.014 U/mg proteins i.e. a decrease of approximately 45% as compared to control.

**Serum (Fig. 3)**

The LDH activity observed in control mice serum was 0.024±0.001 U/mg protein. Enzyme activity of 0.030±0.003 U/mg was seen after 4 hr, 0.033±0.005 after 8 hr and 0.033±0.004 after 20 hr of clenbuterol treatment. There was an enhancement of 25% in LDH activity after 4 hr, 37% after 8 hr and 20 hr of clenbuterol administration. Utmost stimulation in the expression of total LDH was observed at 48 hr i.e. 0.043±0.007 U/mg protein, which accounted for 79%.

**Electrophoresis**

**Ventricles**

Ventricular homogenates of control mice demonstrated three isozymes of lactic dehydrogenase predominantly viz; LDH₁, LDH₂ and LDH₃. LDH₄ and LDH₅, made occasional appearance (Fig. 4). LDH₄ and LDH₅ were present in minute concentration (around 12%). LDH₂ was the most predominant isozymes present in maximum concentration of 31%. LDH₁ had the second place (24%) in the order of concentration (Fig. 5). LDH₁ and LDH₂, are the tissue specific isozymes of heart and are helpful in detecting cardiac injury.

Clenbuterol treatment for 4 hr showed stimulation in the levels of ventricular isoforms. Rise of 30%, 40% and more than 50% was detected for LDH₂, LDH₃ and LDH₄ respectively. LDH₁ on the other hand illustrated a slight non significant increase of 2% in its level and an increase of 7% was noticed in the expression of LDH₅ (Fig. 4A,5). After 8 hr stage an amplification of more than 20% and approximately 30% was witnessed in LDH₄ and LDH₃ respectively while LDH₁ and LDH₂ demonstrated a decline which was more pronounced i.e.
almost 20%, in case of LDH (Fig. 4B,5). A diminution in the concentration of LDH isozymes was seen after 20 hr of clenbuterol administration (Fig. 4C,5). A decrease in the expression of aerobic forms i.e. LDH1 and LDH2 was witnessed which accounted for 11% and 26% respectively while LDH3-LDH5 demonstrated a rise. A significant rise of near about 40% was observed for LDH5. 48 hr stage after clenbuterol treatment resulted in all isoforms showing more or less similar decline in their expression (Fig. 4D) i.e. ranging between 19-26%. Maximum fall (26%) was observed in LDH1 and minimum (19%) in LDH5. LDH2, LDH3 and LDH4 showed a decrease of 22%, 23% and 24% respectively (Fig. 5).

**Serum**
Control serum displayed all five isozymes LDH1, LDH2, LDH3, LDH4 and LDH5. LDH5 comprised the maximum proportion i.e. almost 56% followed by LDH4 and LDH3 which formed more than 17% and about 16% of the total LDH. LDH2 represented 9% of total LDH followed by more than 2% of LDH1 (Fig. 6,7).

Serum after 4 hr of drug treatment showed insignificant change in the expression of various isozymes (Fig. 6A) whereas all the isoforms demonstrated an enhanced expression after 8 hr of clenbuterol administration (Fig. 6B). There was a greater increase of 25% in the activity of LDH2 and to lesser extent in LDH1 i.e. 4%. LDH4 and LDH5 revealed equally enhanced activities (Fig. 7). After 20 hr of clenbuterol treatment, aerobic isoforms i.e. LDH1 and LDH2 and anaerobic form LDH5 displayed a spurt in their activity (Fig. 6C). The increase noticed was 44%, 27% and 20% respectively (Fig. 7). After 48 hr, serum displayed highest LDH levels. Highest levels were observed for LDH1 then followed by LDH2 (Fig. 6D). The percent increase was 67% for LDH1 and approximately 40% for LDH2. Other forms showed a fine increase (Fig. 7).

**Electron Microscopy**
Ultrastructural analysis of control cardiac muscle revealed that myofibers were striated. Each myofiber was composed of small structural units separated by dark Z-bands. Z-bands were very prominent. The dark and light bands were clearly differentiated. The myofibers were packed with rows of mitochondria (Fig. 8A, B). Myocyte nuclei were oval in outline with almost uniformly scattered chromatin. Endoplasmic reticulum could be seen in the vicinity of the nucleus (Fig. 8C). Nuclear envelope was double layered and was thrown in to membranous folds (Fig. 8D).
Effect of β-agonists

Clumping of the nuclear chromatin under nucleolemma was quite prominent after clenbuterol treatment. Some active euchromatin was noticed in the centre of the nucleus and the cytoplasm was in degenerating state (Fig. 9A). Degenerating myofibers were visualized along with vacuole formation. Mitochondrial structure was more or less maintained. Some mitochondria showed clear cristae (Fig. 9B). Clenbuterol caused fading of the nuclear chromatin after 48 hr of its administration. Half of the nucleus was illustrating normal chromatin whereas another half was exhibiting complete fading of chromatin (Fig. 9C). The endoplasmic reticulum showed dilated tubules (Fig. 9D). More pronounced disruption of the outer mitochondrial membrane was reported at 48 hr stage, which normally limited the organelle from other sarcoplasmic contents. Disappearance of mitochondrial membrane exposed cristae (Fig. 9E). Myofibers showed degeneration and mitochondrial shapes were found to be altered at this stage (Fig. 9F).

Figures

Figure 1: Mice ventricular tissue (L.S.).
A: control mice heart ventricles showing normal distribution of cardiomyocytes with central nuclei (N), peripheral fibroblast (F) and branching fibers.

B: Four hr post clenbuterol administration illustrating long branched myocardial fibers with central nuclei and fibroblast nuclei. At some places nuclei are completely absent in the fibers (EF) whereas at another points the fibers have started undergoing myonecrosis (N).

C: After 8 hr of clenbuterol administration demonstrating disturbed arrangement of the myocardial fibers and fibroblasts (F). Enucleated areas (EA) are very prominent.

D: After 20 hr of clenbuterol administration showing massive myonecrosis (N) and macrophage infiltration (MI) around blood vessel (BV) area.

E: After 48 hr post clenbuterol administration badly injured myofibers (N). Nuclei formed a chain like structure (NC) around the degenerating fiber. However completely normal fiber can be spotted in the mid of the section (NF).

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**Figure 2:** Effect of acute dose of clenbuterol (20 mg/kg b. w.) on total mice ventricular lactate dehydrogenase enzyme concentration (Units/mg protein). Values are Mean ± S.E.M. (n=6). P < 0.05*.

**Figure 3:** Effect of acute dose of clenbuterol (20 mg/kg b. w.) on total mice serum lactate dehydrogenase enzyme concentration (Units/mg protein). Values are Mean ± S.E.M. (n=6). P < 0.05*.
Figure 4: Native PAGE (7.5%), demonstrating distribution of lactic dehydrogenase (LDH) isozymes in control mice heart ventricles and clenbuterol (20 mg/kg b. w.) treated ventricles after 4 hr (A), 8 hr (B), 20 hr (C) and 48 hr (D). Ventricles expressed all five isoforms ranging from LDH₁ to LDH₅. An increase in the expression of LDH₃, LDH₄ and LDH₅ was evident except for 48 hr stage. LDH₁ and LDH₂ though experienced enormous decrease in the tissue levels.
Lane 1: Control
Lane 2: Clenbuterol treated.

Figure 5: Percent increase or decrease in the levels of various LDH isozymes of mice ventricles after treating with clenbuterol.
Figure 6: Native gel electrophoresis (7.5%), demonstrating distribution of lactic dehydrogenase (LDH) isozymes in control mice serum and clenbuterol (20 mg/kg b. w.) treated serum after 4 hr (A), 8 hr (B), 20 hr (C) and 48 hr (D). Serum is demonstrating increased levels of almost all the isoforms at one or other stage. LDH₅ expressed to greatest extent at 20 hr stage whereas LDH₁ and LDH₂ showing peak at 48 hr stage.
Lane 1: Control
Lane 2: Clenbuterol treated.

Figure 7: Percent increase or decrease in the levels of various LDH isozymes of mice serum after treating with clenbuterol.
Figure 8: A: Transmission electron photomicrographs (TEM) of control mice heart (longitudinal sections) showing normal myofibrils (F) with prominent Z-bands (Z), intermyofibrillar space (IS) and mitochondria (M).
B: High magnification longitudinal section of control heart displaying structural unit of muscle fibril, sarcomere (S) extending between two Z-lines (Z) and embedded mitochondria (M).
C: Transmission electron photomicrographs (TEM) of control mice heart depicting normal nucleus of the cardiomyofibril with prominent chromatin and well developed endoplasmic reticulum.
D: High magnification view of control mice heart displaying double layered nuclear envelope produced into membranous folds. N indicates nucleus, SER (smooth endoplasmic reticulum), NE (nuclear envelope), RER (rough endoplasmic reticulum) and M (Mitochondria).
Figure 9: A: Cardiomyocyte nucleus (N) demonstrating clumping of the chromatin (heterochromatin) below nuclear envelope (→) after 20 hr of clenbuterol treatment. Some active euchromatin was also visible. Sarcoplasm was undergoing degeneration (D). Normal (M) and, swollen mitochondria (SM) are clearly seen.

B: Mice heart after 20 hr of clenbuterol administration showing less dense mitochondrial matrix (CM) and vacuoles (V). Myofibrils (F) are showing signs of degeneration (D). Mitochondria with intact structure (M) are also present.

C: After 48 hr of clenbuterol administration mice heart exhibiting nucleus (N) of cardiomyocyte with half faded nuclear chromatin (FC) and half normal.

D: Endoplasmic reticulum (ER) in the heart is demonstrating dilation after 48 hr of clenbuterol administration. Nucleus (N) is also seen in the neighbourhood.

E: After 48 hr treatment of clenbuterol cardiac mitochondria structure is severely disrupted. Excessive mitochondrial edema (M) is noticed and many mitochondria displayed disruption of outer membrane (DM). Cristae (C) in such mitochondria are clearly seen. (N) stands for nucleus.

F: Electronogram of mice heart after 48 hr stage exhibiting degenerated areas (D). Mitochondria with altered shapes (AM) are also observed. F represents myofibril, Z indicates Z-line whereas M stands for mitochondria with normal morphology.
DISCUSSION

Clenbuterol is a bronchodilator which is essentially a steroid, but its side effects are as long time thermogen or fat burner,[9, 10] because it elevates resting heart rate and increases internal body temperature. It is known to quickly make people drop weight and burn off fat, because it increases the rate at which calories are burnt, even when a person is not being physically active.

Earlier we evaluated the cytotoxic effects of β-agonists in mice heart and gastrocnemius.[11, 12] The effects of isoproterenol on mice heart and gastrocnemius were undeniable,[11,12] but same could not be deduced in case of clenbuterol. Therefore present study was designed to measure the cytotoxic potential of an acute dose of β-agonist, clenbuterol on mice ventricular tissue in particular, employing additional parameters. The cardiac tissue cells showed clenbuterol induced injury in the form of fiber degeneration, assembly of polymorphonuclear chains around them and widespread nuclear pycnosis. The ultrastructural observations of cardiac muscle revealed mitochondrial swelling followed by disruption and disappearance of cristae after β-agonist administration. This appearance was considered “atrophy of the striated muscle”. Though atrophy, hypertrophy and fibrosis are said to be the means of cell adaptation to a stress,[13] the level of tissue necrosis is worth noticing at the same time. Burniston et al. also observed myocyte necrosis in rat heart at a dose of 5 mg clenbuterol/kg b.w of rats.[14] These researchers confirmed that maximum necrosis in rat heart existed 2.4 mm above the ventricular apex. The major mitochondrial pathology in present study was edema leading to disruption, and is considered as the main ultra structural sign of cellular injury.[15]

Disrupted mitochondrial structure may interfere with the cells ability to utilize oxygen to generate adequate amount of ATP.[16] This in turn may impair the ability of cell to utilize the nutrients to synthesize structural and functional proteins necessary for maintaining the cell. Moreover, reduction of ATP can also shift the metabolism towards anaerobic glycolysis. In addition of being less efficient in terms of energy production, glycolysis is also accompanied by the accumulation of inorganic phosphate and lactic acid which makes the pH inside the cell more acidic. This acidosis can interfere with enzyme functioning and can damage nuclear DNA thus leading to cell death.[17] This has previously been studied in developing brain.[16]

Cell death is known to take place by two pathways i.e. necrosis and apoptosis. An earlier study illustrated that tissue necrosis characteristically involves foci containing many cells that swell (especially the mitochondria and endoplasmic reticulum) because they lose their ability
to maintain their fluid and electrolyte balance. In contrast the mitochondrial morphology is normal in apoptosis just the nuclear chromatin forms clumps at the nuclear periphery. In the current study, some cells showed fading of the nuclear chromatin similar to findings of Kroemer and Goldstein. These workers pointed that nuclear shrinkage and fading are the signs of necrosis. This fading was named karyolysis. Some nuclei illustrated aggregation of chromatin beneath the nuclear envelope which is a sign of apoptosis.

Earlier studies established that infarct-like lesions are produced in the myocardium when injected with isoproterenol, an another β-agonist. These lesions are morphologically similar to those of coagulative myocytolysis (COAM) or myofibrillar degeneration. Our study also witnessed similar degeneration in mice myocardium. Milei et al. suggested that myocardial necrosis induced by isoproterenol is probably due to its primary action on the sarcolemmal membrane, followed by stimulation of adenylate cyclase, activation of calcium and sodium channels, exaggerated calcium inflow and excess of excitation-contraction coupling mechanism leading to more energy consumption and cellular death. Myofilamental alterations such as myocytosis and myofibrillar degeneration were also reported in isoproterenol treated rats.

Rubin and Strayer defined cell injury as alterations in cell structure or functioning resulting from any stress that exceeds the ability of the cell to compensate through normal physiological adaptive mechanism. Although stress can cause injury to any biologically important molecule of a cell. Cell membrane is the first and most important target. Once the membrane integrity is lost, it may affect the entire cell structure and function.

Here, the analysis of total lactate dehydrogenase and its isozymes in ventricular tissue well as serum depicted disruption of myocyte membrane by acute dose of clenbuterol but only at later experimental stage i.e. 48 hr. Initially the levels of enzyme were increasing persistently illustrating that the cells are under stress pointing towards increased energy requirements of muscle after β-agonist administration as discussed above. LDH1 was released from the cardiac tissue earlier than LDH2 after ventricular injury, serving as early indicator. Our findings are in accordance with Prabhu et al., who also noted an increased expression of serum LDH isozymes bands especially LDH1 caused by isoproterenol.

Serum LDH1 was enhanced to a level of 44% after 20 hr and 67% after 48 hr of clenbuterol administration. However, LDH2 showed an increase of 27% at 20 hr stage and 40% at 48 hr
stage. Past studies in rats have found that serum LDH activity following, single subcutaneous (s.c.) dose of 5-250 mg isoproterenol/kg b. w. after 2, 4, 6, 8 and 24 hr, drastically increased from 2 hr onwards especially that of LDH$_1$ and LDH$_2$. Maximum increase was observed after 4-8 hr. Present study however recorded maximum increase in serum LDH at 48 hr stage. This could be due to the different route of drug administration which is oral in the present case.

Thus it can be concluded that a single acute dose (20 mg/kg b.w) of β-adrenergic agonist clenbuterol, can result in ventricular aberration in mice. No doubt there were several signs of cell adaptation but bulk of tissue displayed irrevocable disintegration.

**REFERENCES**


