Analysis of isoproterenol-induced changes in gastrocnemius muscle and serum lactate dehydrogenase expression in mice

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ABSTRACT

Introduction: Beta-adrenergic agonists are highly abused by sportspeople for their muscle anabolic and lipolytic effects. Muscle growth effects are thought to result from beta-2 adrenoceptor activation. This study attempted to characterise muscle (gastrocnemius) tissue damage following the administration of isoproterenol.

Methods: Adult male Balb-C mice were treated with a single oral dose of isoproterenol (100 mg/kg body weight) for 4, 8, 20, 48 and 72 hours. Control mice received equal doses of saline. The animals were killed at the respective stages, followed by the collection of gastrocnemius and blood. Serum was then separated from blood. Histopathology and lactate dehydrogenase (LDH) assays were performed.

Results: Beta-adrenoceptor activation-induced histological changes began with structural aberrations, and ultimately resulted in myonecrosis and extensive degeneration within hours of the administration of isoproterenol. The effects of beta-agonist administration on muscle myofibre organisation were visible within four hours and became most prominent at 20 and 48 hours. Augmentation of more than 20 percent in muscle LDH activity was observed at 4, 8 and 72 hour stages, and was accompanied by a significant decline of 19 and 27 percent at 20 and 48 hour time points, respectively. Serum corroborated the above results.

Conclusion: Isoproterenol treatment produced considerable histopathological changes, including myonecrosis in mice gastrocnemius, resulting in a leaky sarcolemma and the release of marker enzyme, LDH into the serum (more evident after 20 and 48 hours). This suggests that isoproterenol promotes the process of necrosis in mice gastrocnemius at the concentration employed, which raises a significant question regarding the use and abuse of beta-agonists.

Keyword: electrophoresis, gastrocnemius muscle, isoproterenol, lactate dehydrogenase, tissue damage

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INTRODUCTION

Different β-adrenergic agonists, including clenbuterol and isoproterenol, have been documented to act as potent growth stimulators and repartitioning agents *in vivo* by increasing body mass and decreasing fat mass, respectively.[1-5] Isoproterenol exhibits agonist activity at both β_1 and β_2 receptors. It has been shown that isoproterenol induces myocardial cell injury similar to the one reported for myocardial infarction, myocardial ischaemia, cardiac stress and chagasic cardiomyopathy.[6] Several studies have investigated the molecular-cellular mechanisms of isoproterenol-induced cell injury of the myocardium.[7]

Many enzymes are released into the serum in case of cell injury. Such enzymes are known as marker enzymes. The present study takes into account lactate dehydrogenase (LDH) as a marker of cell injury. Many instances of high doses of isoproterenol causing cell injury have been reported, but in all these cases, subcutaneous or intraperitoneal administration of this β-agonist had been used. There is, however, no evidence of oral ingestion of the drug, which is the most common and economical route of drug administration. Our study aimed to determine the level of cell injury in mice gastrocnemius using a single high oral dose of isoproterenol.

METHODS

Adult male Balb-C mice (20–25g) (Central Research Institute, Kasuali, Himachal Pradesh, 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 were approved by the Institutional Animal Ethics Committee of the University. Isoproterenol hydrochloride (Sigma Aldrich, St Louis, MO, USA) was used as a pure chemical substance. The mice were reared in the animal house of the department for a month. Normal healthy mice that showed no sign of morbidity were divided into two groups. Mice in the first group served as controls and received saline, while those in the second group were treated with a single high oral dose of isoproterenol (100 mg/kg body weight). The effects of this β-adrenergic drug were seen after 4, 8, 20, 48 and 72 hours of treatment.

The gastrocnemius muscle was homogenised in 0.2 M Tris HCl buffer, pH 7.3. The homogenate was centrifuged at 4,000 rpm, and supernatant was either immediately employed for electrophoretic separation or stored in glycerol (1.0:0.25 v/v). Serum samples were prepared by keeping the blood as such overnight at 8˚C. Blood cells were coagulated and the serum was separated as a distinct layer. Protein concentration was determined by the method employed by Lowry et al.[8] Tissue blocks of gastrocnemius were immediately fixed in aqueous Bouin's fixative for 24 hours, and subsequently washed in water, dehydrated, cleared in xylene and embedded in paraffin wax (58ºC to 60ºC). 5-µm thin sections were cut on a Spencer type rotary microtome and subjected to haematoxylin and eosin staining. Paraffin-embedded muscle sections were dewaxed in xylene, and hydrated, stained and dehydrated in different grades of alcohols. They were then mounted in DPX and photographed using a Leica DM LS2 photomicroscope that was equipped with an automatic DFC C32 camera (Leica Microsystems, Wetzlar, Germany).

The total LDH activity in gastrocnemius and serum was determined by employing a standard assay, as proposed by Borgmann et al.[9] Reaction velocity was determined by a decrease in absorbance at 340 nm following the oxidation of nicotinamide adenine dinucleotide (NADH). Change in absorbance was finally converted into units of LDH activity. One unit of LDH activity was considered to be 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. Absorbance was recorded using a Hitachi 150-20 spectrophotometer (Hitachi Ltd, Tokyo, Japan).

LDH isozymes were resolved on a 1-mm thick slab gel according to the method described by Cooper.[10] Proteins were estimated according to the method of Lowry et al,[8] and equal amounts of proteins from the particular tissue were loaded in different lanes of the gel. Different isozymes of LDH were resolved on 7.5% gel, employing native polyacrylamide gel electrophoresis. Tris glycine buffer (pH 8.3) was used as a running buffer. Electrophoretically resolved LDH isozymes were finally visualised 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 PMS and 100 mM sodium lactate (pH 7.0). Gels containing resolved isozymes were kept in the incubation medium for 20 minutes at 37ºC. Finally, the gels were fixed in 5% acetic acid, dried and photographed. Different LDH isozymes appeared as dark (blue/violet) bands. Both tissue homogenates and serum samples were employed in electrophoretic separation of LDH isozymes. Quantitative electrophoresis (densitometry) was performed to evaluate the altered isozymic expression after isoproterenol administration. Data was expressed as mean ± standard error of mean. Statistical significance was determined by the application of one-way ANOVA, followed by post hoc Dunnett's test, in order to find the mean difference between the groups. The differences were assumed to be significant at $p < 0.05$.

RESULTS

In the gastrocnemius of control mice, the myofibres were of relatively uniform size and shape, and fit into each other in a mosaic pattern (Fig. 1a). The fibres that were in direct contact with each other remained separated by a very thin, almost invisible layer of connective tissue known as endomysium. The myofibre nuclei were located at the periphery of the cells and the cytoplasm was fairly uniformly distributed (Fig. 1a).

In mice that were administered isoproterenol, the myofibers could be seen individually after four hours, which indicated that the connective tissue binding them, i.e. the endomysium, was missing in such places. The characteristic feature seen in the gastrocnemius of control mice, (i.e. the mosaic pattern), was distorted to some extent (Fig. 1b); the myofibres lost their normal outline and became rounded or atrophic, while the perimysium underwent huge degeneration. Some hypercontracted opaque fibres with dark stain were noted. At a few points, macrophage infiltration was also observed around myofibres that might be undergoing myophagocytosis (Fig. 1b). One or two such myofibres in the section had undergone such extensive hypertrophy that they had started splitting. These fibres were used as a tool in the muscle pathology to determine which muscle fibres were

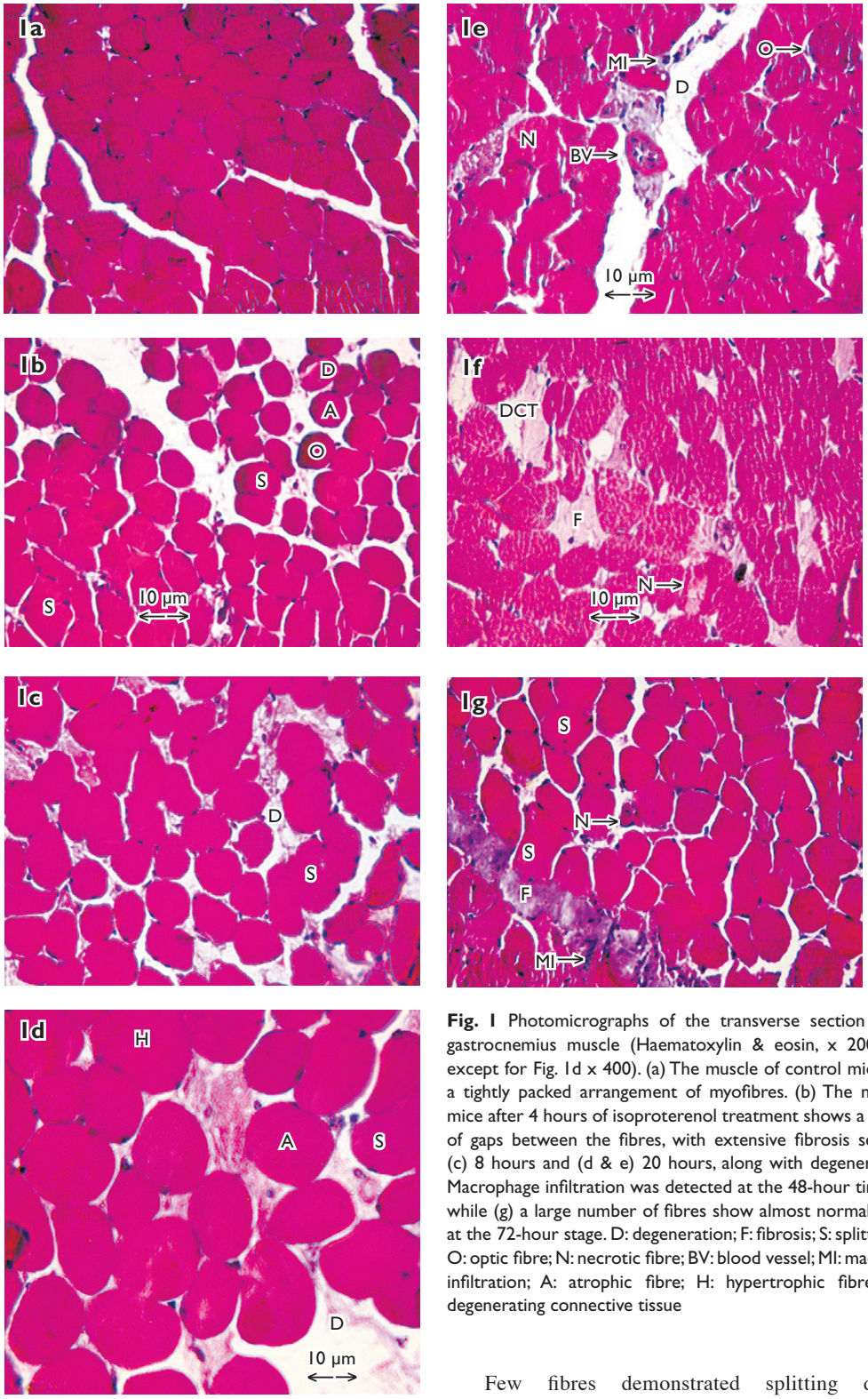

Fig. 1 Photomicrographs of the transverse section of mice gastrocnemius muscle (Haematoxylin & eosin, x 200 for all, except for Fig. 1d x 400). (a) The muscle of control mice shows a tightly packed arrangement of myofibres. (b) The muscle of mice after 4 hours of isoproterenol treatment shows a widening of gaps between the fibres, with extensive fibrosis seen after (c) 8 hours and (d & e) 20 hours, along with degeneration. (f) Macrophage infiltration was detected at the 48-hour time point, while (g) a large number of fibres show almost normal outlines at the 72-hour stage. D: degeneration; F: fibrosis; S: splitting fibre; O: optic fibre; N: necrotic fibre; BV: blood vessel; MI: macrophage infiltration; A: atrophic fibre; H: hypertrophic fibres; DCT: degenerating connective tissue

under stress, as atrophy and hypertrophy were means of adaptation to cell injury. After eight hours of isoproterenol administration, the endomysial connective tissue started undergoing fibrosis and degeneration in some places. The normal mosaic plan of the muscle was completely disturbed, and the majority of the fibres had become rounded, i.e. they were going through atrophy.

Few fibres demonstrated splitting due to hypertrophy of the fibers, which was not considered a normal phenomenon. There was evidence of macrophages assembling around a fibre, indicating inflammation (Fig. 1c). The appearance of the muscle fibres had changed completely by the 20th hour of drug treatment, with mostly prominent round atrophic fibres seen. Fibre splitting was clearly noticeable, and fibrosis was not uncommon (Fig. 1d). Severe inflammation

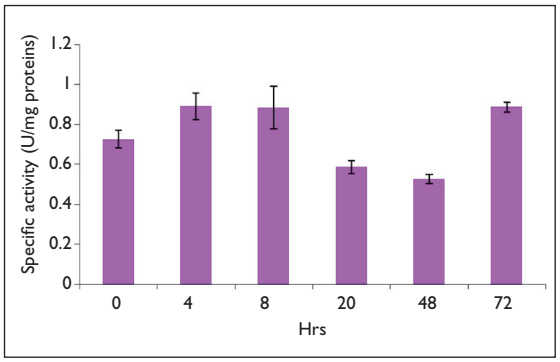

Fig. 2 Graph shows the effect of a single high dose of isoproterenol (100 mg/kg body weight) on mice gastrocnemius total LDH concentration.

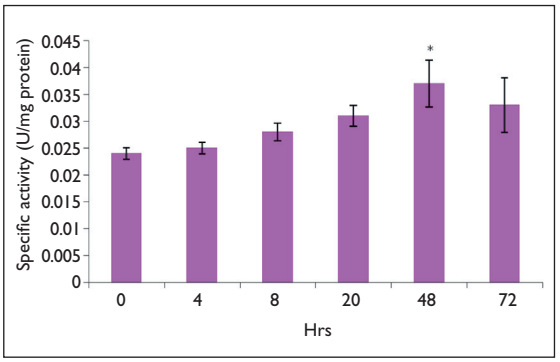

Fig. 3 Graph shows the effect of a single high dose of isoproterenol (100 mg/kg body weight) on mice serum total LDH concentration.

and degeneration could be seen in the muscle section. Macrophage infiltration was accompanied by perimysial degeneration. Myofibres undergoing necrosis were also visible (Fig. 1e). Fibrosis was most evident at the 48-hour stage, with a large number of round atrophic fibres. Necrotic fibres were also evident. At many time points, the connective tissue was noted to have undergone degeneration (Fig. 1f). Considering all stages of the experiment, the 72-hour stage was found to be comparable to the four-hour stage. It was difficult to determine endomysial fibrosis, but perimysium was highly fibrotic and was accompanied by a large number of macrophages. However, the gap between fibres was not a normal feature. There were few necrotic, round or splitting fibres, with the majority comprising normal outlines with a tendency toward a normal mosaic structure (Fig. 1g).

The total LDH activity observed in control mice gastrocnemius was 0.725 ± 0.043 U/mg proteins. The gastrocnemius demonstrated an augmentation in LDH concentration immediately after isoproterenol administration. The activity seen after four hours of isoproterenol treatment was 0.890 ± 0.066, i.e. an increase of 23% as compared to the control group. The eight-hour stage also witnessed almost identical results (0.884 ± 0.107). On the contrary, after 20 and 48 hours, the LDH activity showed a decline of 19% (0.586 ± 0.032) and 27% (0.525 ± 0.022), respectively. The LDH activity at the 72-hour stage showed enhanced levels (0.886 ± 0.024) of LDH enzymes (Fig. 2).

Serum, on the other hand, displayed the reverse pattern. LDH activity observed in control mice serum was 0.024 ± 0.0011 U/mg proteins. Negligible changes in LDH activity (0.025 ± 0.011) were observed in the isoproterenol-treated mice after four hours, and to some extent, after eight hours (0.028 ± 0.0016). At 20 hours post drug administration, the serum demonstrated an amplification of nearly 30% at 0.031 ± 0.002. The activity continued to increase until the 48-hour stage, and was observed to be 0.037 ± 0.004. A total enhancement of 54% was detected ($p < 0.05$). LDH level declined after 72 hours of drug treatment (0.033 ± 0.005), although the values were still higher than that of the controls (Fig. 3). Native gel electrophoresis of control gastrocnemius displayed five isozymes, LDH_1, LDH_2, LDH_3, LDH_4 and LDH_5. The anaerobic isoforms LDH_5 comprised the maximum proportion, i.e. more than 72%, followed by LDH_4, which formed about 11% of the total LDH. LDH_3 and LDH_2 represented 6% of total LDH, followed by LDH_1, which constituted almost 5% of the total gastrocnemius LDH content. There was a spurt in the activity of anaerobic isoforms after four hours of isoproterenol treatment (Fig. 4a). An increase of 10% in LDH_5 was observed. LDH_4 also showed a microscopic rise of 5%. Other isozymic forms displayed the reverse pattern by showing a decrease in their activities. LDH_1, LDH_2 and LDH_3 demonstrated a decline of 31%, 20% and 3%, respectively (Fig. 5). After eight hours, all the aerobic isozymes showed a spurt in their levels (Fig. 4b). LDH_1 demonstrated an activity of around 41% higher than that of the control.

Similarly, LDH_2 exhibited an increase of 25% in its activity. At the same time, LDH_3 displayed an increase, with 57% higher levels than that of the control. The levels of anaerobic forms were high, but the increase was found to be on the lower side when compared with aerobic forms. Both LDH_4 and LDH_5 illustrated a stimulation of around 17% each (Fig. 5). Aerobic isozyme levels were higher even after 20 hours of isoproterenol administration (Fig. 4c), although the rise was lesser than that at the eight-hour stage. Greater stimulation was seen for LDH_2 and LDH_1 at 31% and 27%, respectively. LDH_3 levels also demonstrated an increase of 18%. On the other hand, a decrease in activities was noted in the anaerobic forms.

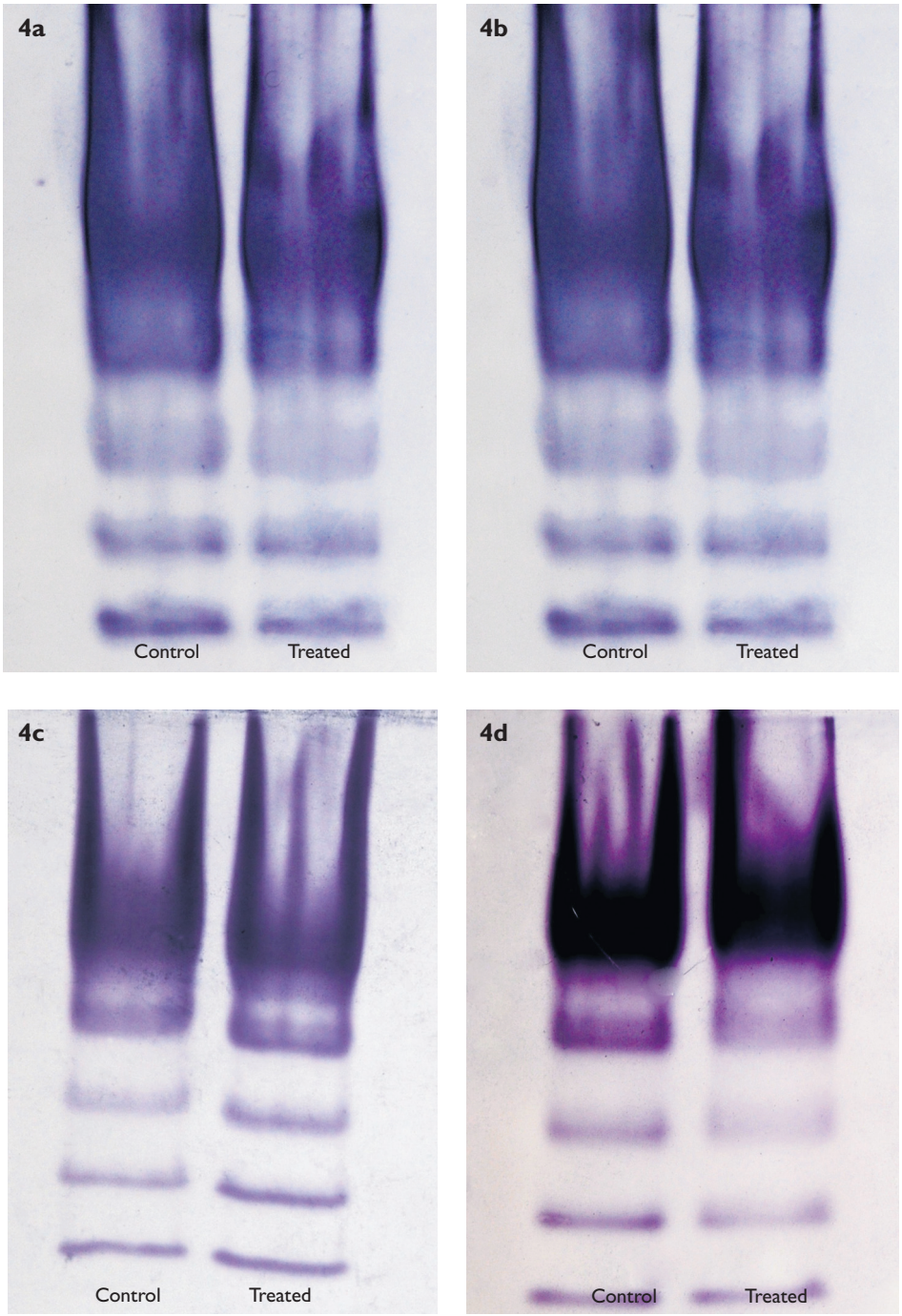

Fig. 4 Native gel electrophoresis (7.5%) shows the distribution of LDH isozymes in control mice and isoproterenol-treated gastrocnemius after (a) 4 hrs; (b) 8 hrs; (c) 20 hrs; and (d) 48 hrs. Anaerobic forms (LDH_5 and LDH_4) initially witnessed a burst but their levels were depressed at later stages. Aerobic forms (LDH_1 and LDH_2) were also stimulated significantly, but a massive fall in their activities was seen at 48 hrs.

LDH_5 showed a fall of 12%, followed by a decline of 7% in LDH_4 levels (Fig. 5). At the 48-hour stage, we witnessed a decline in the levels of all the isozymic forms of LDH (Fig. 4d). The most predominant decline (65%) was observed in the levels of LDH_2. This was followed by LDH_4 (18%), LDH_1 (14%), LDH_3 (13%) and LDH_5 (6%) (Fig. 5).

Serum also displayed five isozymes, LDH_1, LDH_2, LDH_3, LDH_4 and LDH_5. LDH_5 comprised the maximum proportion, i.e. almost 56%, followed by LDH_4 and LDH_3, which formed more than 17% and about 16% of the total LDH, respectively. LDH_2 represented 9% of the total LDH, followed by > 2% of LDH_1. LDH_1 levels were seen to decline after four hours (Fig. 6a); a 32% decline was noted. At this point, LDH_2 expression also showed a reduction of 22%, while LDH_4 and LDH_5 showed an increased expression of 22% and 19%, respectively, and LDH_3 showed a minute increase of 7% (Fig. 7).

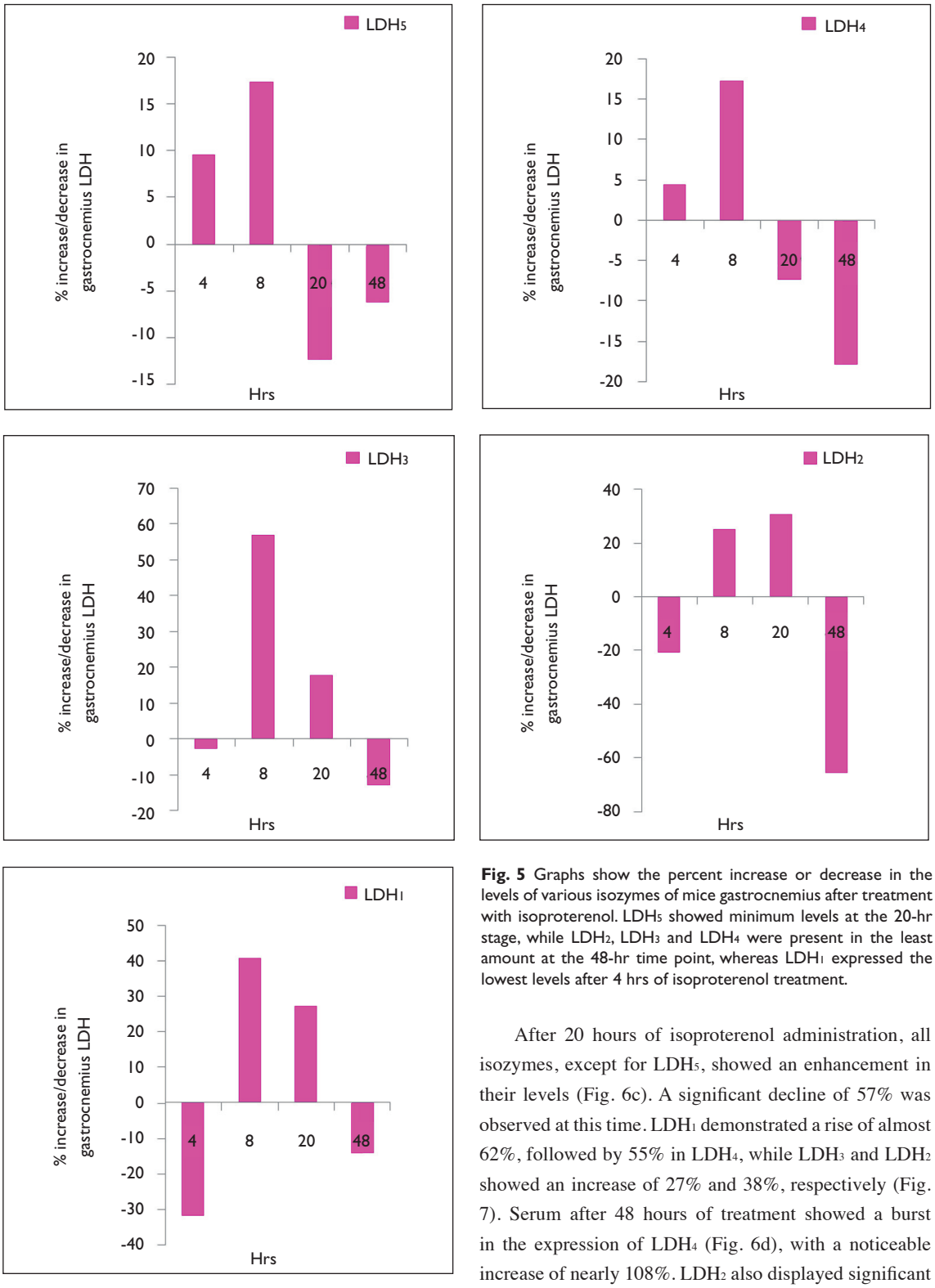

Fig. 5 Graphs show the percent increase or decrease in the levels of various isozymes of mice gastrocnemius after treatment with isoproterenol. LDH_5 showed minimum levels at the 20-hr stage, while LDH_2, LDH_3 and LDH_4 were present in the least amount at the 48-hr time point, whereas LDH_1 expressed the lowest levels after 4 hrs of isoproterenol treatment.

Isoproterenol resulted in an increase in the expression of all LDH isozymes after eight hours of its administration (Fig. 6b). Extreme enhancement of 55% was observed in the levels of LDH_1, while LDH_2 and LDH_3 showed an increase of up to 42% and 44%, respectively. The enhancement observed in LDH_4 and LDH_5 was lower, at 10% and 6%, respectively (Fig. 7).

After 20 hours of isoproterenol administration, all isozymes, except for LDH_5, showed an enhancement in their levels (Fig. 6c). A significant decline of 57% was observed at this time. LDH_1 demonstrated a rise of almost 62%, followed by 55% in LDH_4, while LDH_3 and LDH_2 showed an increase of 27% and 38%, respectively (Fig. 7). Serum after 48 hours of treatment showed a burst in the expression of LDH_4 (Fig. 6d), with a noticeable increase of nearly 108%. LDH_2 also displayed significant stimulation at 72%. The levels of LDH_3 and LDH_5 increased by 59% and 37%, respectively. LDH_1, however, showed a considerable decline of 37% (Fig. 7).

DISCUSSION

Synthetic β-adrenoceptor agonists such as isoproterenol, clenbuterol and fenoterol were initially developed for acute asthma treatment to facilitate bronchiolar smooth

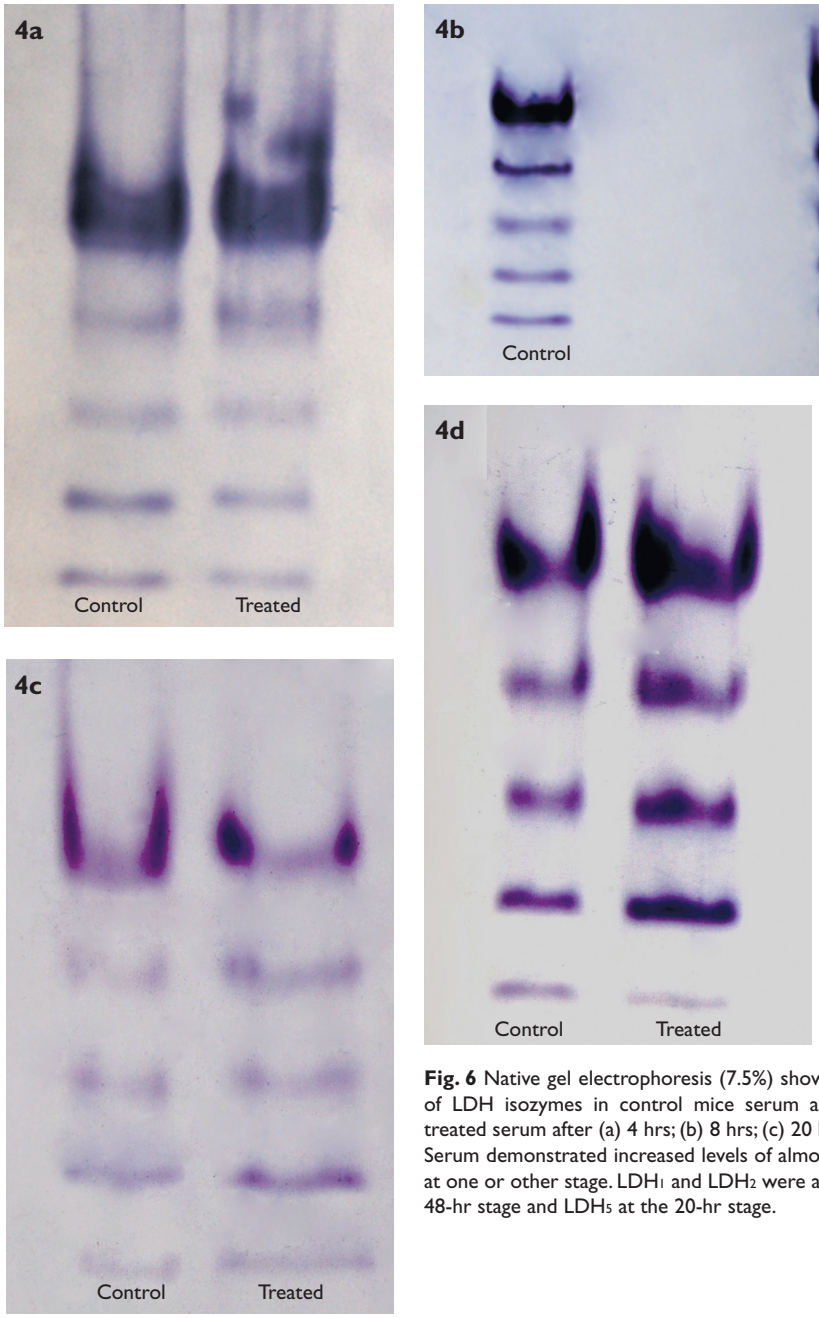

Fig. 6 Native gel electrophoresis (7.5%) shows the distribution of LDH isozymes in control mice serum and isoproterenol-treated serum after (a) 4 hrs; (b) 8 hrs; (c) 20 hrs; and (c) 48 hrs. Serum demonstrated increased levels of almost all the isoforms at one or other stage. LDH_1 and LDH_2 were at their peak at the 48-hr stage and LDH_5 at the 20-hr stage.

muscle dilation.[11] Further investigations revealed that β-agonists have an anabolic effect on skeletal muscle when administered at higher doses.[12] They increased muscle cell growth and protein content both *in vitro*[13] and *in vivo*,[14] as well as in peripheral skeletal muscles in normal rats. Skeletal muscles contain predominantly β_2 receptors, and growth effects are thought to be the result of β_2-adrenoceptor activation, with increased cyclic adenosine monophosphate levels.[15,16] Similar effects were also found in pathological situations, including denervation.[17] Histological examination of gastrocnemius in the present study showed hypertrophy, which implied muscle anabolic effects. Hypertrophy is also a means of adaptation after cell injury.

We measured the levels of isoproterenol cytotoxicity in mice after treatment with a single high dose isoproterenol (100 mg/kg body weight). From previous studies, it is clear that isoproterenol is capable of causing cell injury.[18] Histopathological observation done in our study also indicated tissue damage after treatment with isoproterenol. The various changes observed included the loss of a tight-packing arrangement (a characteristic of control muscle), fibrosis, atrophy, hypertrophy and fibre degeneration accompanied by infiltration of macrophages. These changes were especially prominent after 20 and 48 hours of isoproterenol administration. Assay for LDH, a cytosolic enzyme and a well-known marker of cell injury, was performed to confirm these changes in cellular structure and function.

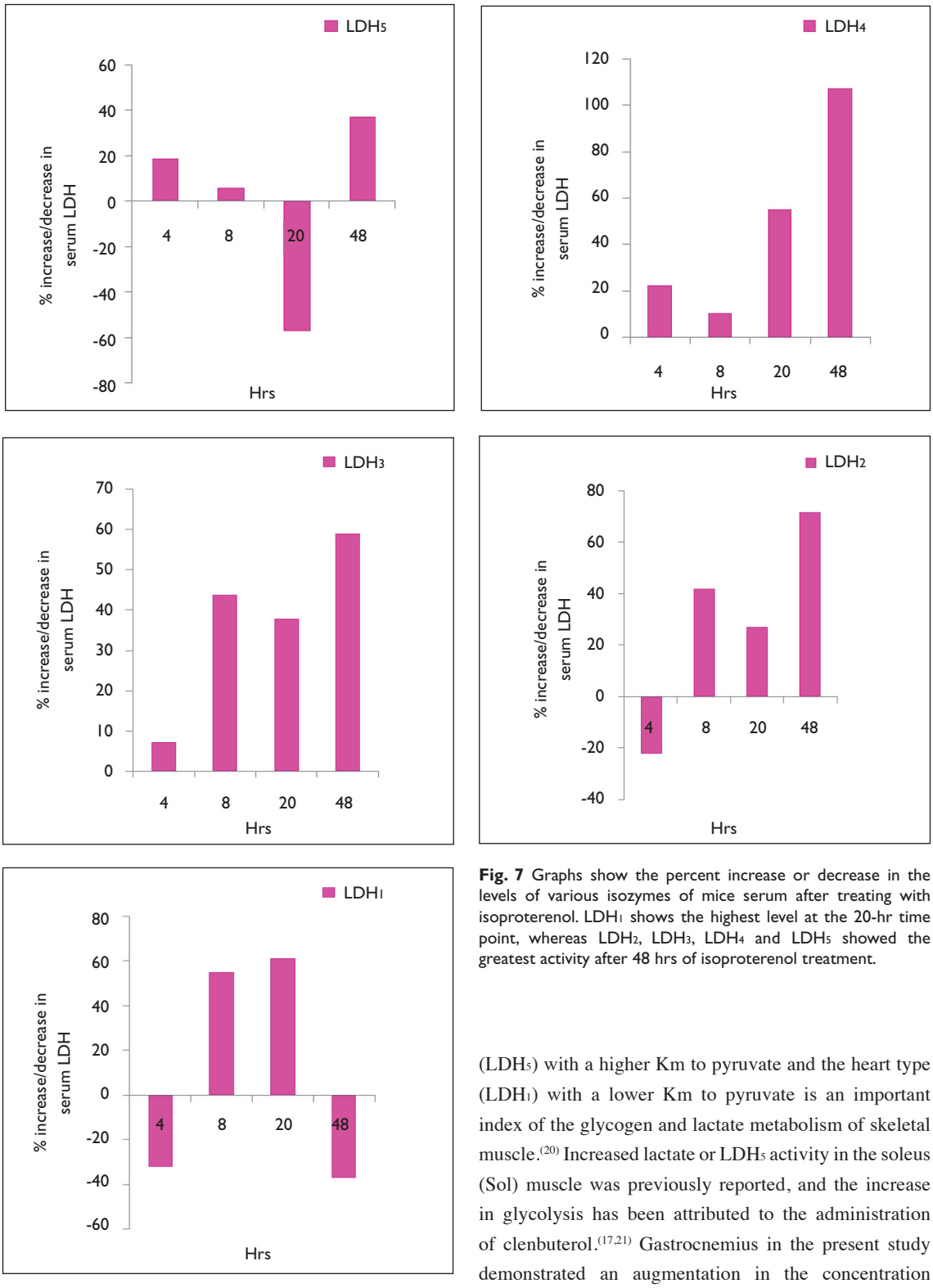

Fig. 7 Graphs show the percent increase or decrease in the levels of various isozymes of mice serum after treating with isoproterenol. LDH_1 shows the highest level at the 20-hr time point, whereas LDH_2, LDH_3, LDH_4 and LDH_5 showed the greatest activity after 48 hrs of isoproterenol treatment.

Gastrocnemius is an anaerobically active tissue that depends on glycolysis for its energy needs. LDH converts the final product of glycolysis (pyruvate) to lactate in the scarcity of oxygen, which acts as a temporary reservoir of H^+. Thus, LDH plays a pivotal role in anaerobic glycolysis.[19] The ratio between the muscle-type isozyme (LDH_5) with a higher Km to pyruvate and the heart type (LDH_1) with a lower Km to pyruvate is an important index of the glycogen and lactate metabolism of skeletal muscle.[20] Increased lactate or LDH_5 activity in the soleus (Sol) muscle was previously reported, and the increase in glycolysis has been attributed to the administration of clenbuterol.[17,21] Gastrocnemius in the present study demonstrated an augmentation in the concentration of total LDH immediately after the administration of isoproterenol. Enhancements of more than 20% were noticed after each of the four-, eight- and 72-hour stages. Biochemical results were further complemented with electrophoretic studies of LDH isozymes. LDH_5 expression rose after four and eight hours of isoproterenol administration.

Serum levels of creatine kinase and LDH are the

diagnostic indicators of many pathological conditions. Many studies have found that an increase in the activity of these enzymes in serum is due to the leakage of enzymes from tissues after injury. Manjula et al found similar results after isoproterenol-induced necrosis in the heart.[22] Our study also found a significant decline of 19% and 27% in muscle total LDH activity after isoproterenol administration, with corresponding high serum LDH levels at the 20-hour (30%) and 48-hour (54%) stages, respectively. Serum LDH_5, on the other hand, showed a decline in its expression after 20 hours of isoproterenol treatment, which is ambiguous. A massive enhancement in serum LDH_5 expression was noticeable at the 48-hour stage, which is of great diagnostic importance.

The initial rise in muscle LDH levels at four and eight hours may indicate increased energy requirements of the muscle after isoproterenol administration, resulting in enhanced glycolysis and the production of pyruvate, which caused the enhanced LDH activity to convert it to lactate. As is evident from the electrophoretic results, the aerobic isozymes in gastrocnemius showed a decline at the initial stages of the experiment. There is a shift in the isozymic expression from aerobic to anaerobic. However, at the eight-hour stage, a spurt in the activity of aerobic as well as anaerobic isozymes was observed. This pointed toward extremely high-energy metabolism, as lactate is not only an end product of glycolysis and glycogenolysis, but also a substrate for oxidation and gluconeogenesis.[23] The decline in LDH levels after the 20-hour and 48-hour stages is acceptable because LDH, as expected, would be released from the muscle into serum if isoproterenol had caused damage to the cell membrane or sarcolemma.

From the present study, we conclude that a single oral dose of isoproterenol 100 mg/kg body weight results in enhanced muscle LDH activity in mice initially, but causes muscle damage after 20 and 48 hours of drug treatment. Serum corroborated the above findings by showing high LDH levels.

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