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Dose-dependent separation of the hypertrophic and myotoxic effects of the β_2 -adrenergic receptor agonist clenbuterol in rat striated muscles.

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Abstract

Muscle growth in response to large doses (i.e., mg.kg⁻¹) of β_2 -adrenergic receptor agonists has been consistently reported. However, such doses may also induce myocyte death in the heart and skeletal muscles and hence may not be applicable safe doses for humans. Here, we report the hypertrophic and myotoxic effects of different doses of clenbuterol. Rats were infused with clenbuterol (range, 1 μ g to 1 mg.kg⁻¹) for 14 days. Muscle protein content, myofiber cross-sectional area and myocyte death were then investigated. Infusions of 10 μ g.kg⁻¹.d⁻¹ of clenbuterol significantly ($P<0.05$) increased the protein content of the heart (12-15 %), soleus (12 %), plantaris (18-29 %), and tibialis anterior (11-22 %) muscles, with concomitant myofiber hypertrophy. Larger doses (100 μ g or 1 mg) induced significant ($P<0.05$) myocyte death in the soleus (peak 0.2 ± 0.1 % apoptosis), diaphragm (peak 0.15 ± 0.1 % apoptosis) and plantaris (peak 0.3 ± 0.05 % necrosis), and significantly ($P<0.05$) increased the area fraction of collagen in the myocardium. These data show that the low dose of 10 μ g.kg⁻¹.d⁻¹ can be used to investigate the anabolic effects of clenbuterol in the absence of myocyte death.

Keywords

Apoptosis; collagen; heart muscle; hypertrophy; necrosis; skeletal muscle

Introduction

Administration of β_2 -adrenergic receptor (AR) agonists increases muscle growth 25, 30, 38, 39 and results in myofiber hypertrophy 33, 41. The increase in skeletal muscle mass is mediated through the β_2 -AR 10, 18 and arises from an increased rate of protein synthesis 17, 27 and decreased rates of both calcium-dependent 26 and ATP-dependent 7, 40 proteolysis. Consequently, these agents might be useful as an intervention against muscle wasting 7, 33. However, contraindications to the therapeutic use of β_2 -AR agonists exist. For example, studies that have investigated the combined effects of exercise and clenbuterol have revealed an antagonistic relationship between them 13, 19, 21, 34. Duncan et al. 13 reported a relative

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reduction (43 %) in the exercise capacity of rats administered clenbuterol ($2 \text{ mg.kg}^{-1}.\text{d}^{-1}$) in conjunction with exercise training, compared with rats that were only exercised. Furthermore, a number of the endurance-trained animals that were receiving clenbuterol died due to sudden cardiac failure and those animals that did not undergo exercise training had an increased myocardial collagen content. Previous work from our laboratory provides a possible explanation for the detrimental effects of clenbuterol on exercise performance. Like the less selective β -AR agonist, isoprenaline 15, 28, 37, clenbuterol is also able to induce myocyte apoptosis 6 and necrosis 5 in the heart and soleus muscles of laboratory rats. Myocyte death in the heart is mediated through clenbuterol's neuromodulation of the sympathetic system and stimulation of the cardiomyocyte β_1 -AR 6. In contrast, myofiber death in skeletal muscle is mediated by clenbuterol's direct stimulation of the β_2 -AR 6.

Because of clenbuterol's potential to induce myocyte death its use as a therapeutic agent may be limited. Presently, there is abundant data on the growth-promoting effects of large doses (mg.kg^{-1}) of clenbuterol but these may not be appropriate to the proposed safe human dose ($\mu\text{g.kg}^{-1}$). The current work investigates the hypertrophic and myotoxic effects of controlled infusions of different doses (range = $1 \mu\text{g}$ to $1 \text{ mg kg}^{-1} \text{ d}^{-1}$) of clenbuterol. Our working hypothesis is that clenbuterol's desirable hypertrophic effects can be separated from its detrimental myotoxic effects by carefully controlling the dose administered.

Materials and methods

Animal husbandry

All experimental procedures were conducted in accordance with national statutes and local Ethics Committee guidelines. Male Wistar rats were bred in-house in a conventional colony derived from specific pathogen free animals (Bantin & Kingman, Hull, UK). Environmental conditions were controlled at $20 \pm 2^\circ\text{C}$, 45 - 50 % humidity with a 12-h light (0600 - 1800) and dark cycle. Water and food (containing 18.5 % protein) were available ad libitum and daily consumptions, throughout the experimental period, were recorded for each individual animal.

Clenbuterol administration

Five independent groups of animals ($n = 6$ to 9, in each group) were infused with either $1 \mu\text{g}$, $10 \mu\text{g}$, $100 \mu\text{g}$ or $1 \text{ mg.kg}^{-1}.\text{d}^{-1}$ of clenbuterol or the saline vehicle only for 14 days, via subcutaneously implanted osmotic pumps (Alzet, Palo Alto, CA; model 2002). A fresh solution of clenbuterol was prepared in sterile 154 mmol NaCl immediately prior to use and the osmotic pumps prepared using aseptic technique. A weight-matched day 0 control group ($n = 6$) was also included; these animals did not receive any clenbuterol or saline and were not subjected to any surgical procedures. Muscles harvested from the day 0 and saline infused controls were used as a reference to describe normal growth in the absence of clenbuterol.

Surgical procedures

General anesthesia was induced in a chamber using 4 % isoflurane in medical oxygen and subsequently maintained (1.5 %) via a nosepiece. The animal's body temperature during anesthesia was maintained using a heated mat. An incision was made $\sim 10 \text{ mm}$ lateral to the lumbar spine and a subcutaneous pocket opened by blunt dissection. An osmotic pump was placed in the pocket and the incision closed using three discontinuous stitches. Typically the entire surgical procedure from induction through to recovery took less than 15 minutes to complete. Surgery was conducted using an aseptic technique and it was not necessary to use prophylactic antibiotics or postoperative analgesia. After infusion for 14 days with either

clenbuterol or saline, the animals were killed and the heart, diaphragm, soleus, plantaris and tibialis anterior muscles harvested.

Muscle processing

The atria and great vessels of the heart were removed directly superior to the coronary sulcus and the ventricles mounted apex uppermost. A standardized segment of the diaphragm, taken parallel and immediately anterior to the right external branch of the phrenic artery, and a segment of the mid-belly from each hindlimb muscle were mounted in transverse section and supported with blocks of liver. Tissues were then snap-frozen in supercooled isopentane and stored at -80°C . Cryosections ($5\mu\text{m}$) were cut from each muscle and stored at -20°C . Cryosections from the heart were taken to coincide with the previously determined 5, 6 topographical region (2 mm from the apex) of peak cardiomyocyte death. The contralateral skeletal muscles (tibialis anterior, plantaris and soleus) to those used for histology, were frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

Muscle histology

Hematoxylin and eosin staining was used for routine qualitative assessment of muscle composition both of control and experimental tissues. Picric sirius red histochemistry was used to investigate changes in collagen distribution in cardiac and skeletal muscle, essentially using the method of Sweet et al. 36. Myocyte death was detected immunohistochemically. Apoptosis was identified using an anti-caspase 3 antibody (Ab; R&D systems, Minneapolis, MN), described previously in detail and verified with dUTP nick-end labelling and annexin V 6, 15. Myocyte-specific necrosis was detected using an anti-myosin Ab ($0.3\text{ mg}\cdot\text{kg}^{-1}$) administered (i.p.) 12 h before the animals were killed, as previously described 5, 28, 37.

The incidence of cardiomyocyte death was quantified from six to eight fields of view ($\times 100$ magnification), encompassing the entire subendocardial region (approximately 10^4 cells) of the heart. Positive staining (apoptosis or necrosis) was differentiated from the hematoxylin background and quantified, as a percentage of the total area, using image analysis (Lucia; LIM, Hostivar, Czechoslovakia). To quantify myocyte death in the skeletal muscles, three random fields of view ($\times 100$ magnification) across each transverse section were digitized. Both injured and viable myofibers were counted (>700), and the number of damaged fibers expressed as a percentage of the total.

To investigate myofiber hypertrophy, skeletal muscle cryosections were exposed to primary antibodies (Sigma, Poole, UK) specific for either type I or type II myosin heavy chains (MHC) and Ab binding visualized using a standard immunoperoxidase technique (Vectastain; Vector Laboratories, Burlingame, CA, USA). Muscle cryosections were viewed ($\times 100$ magnification) by light microscopy and image analysis software (Lucia) used to measure the fiber cross-sectional areas (CSA). One hundred myofibers that stained positive for predominantly either type I or II MHC (200 myofibers in total) were measured from 5 separate fields of view across each muscle cryosection.

Biochemical analysis

Frozen skeletal muscles or a portion of the myocardium were pulverized in liquid nitrogen using a mortar and pestle. An accurately weighed portion ($\sim 100\text{ mg}$) of this powder was homogenized on ice, using a PolyTron homogenizer, in 10 volumes of (in mmol) 100 NaCl, 50 Tris, 2 EDTA, 0.5 dithiothreitol pH 7.5 at 4°C plus Complete protease inhibitor (Roche Diagnostics, Lewes, UK). The protein concentration of a $5\mu\text{l}$ aliquot of this homogenate was measured using a modified microtiter plate version of the Bradford assay (Sigma, Poole, UK). The total protein content of each muscle was then calculated by multiplying protein

concentration, homogenate volume, and the fraction of the ground portion relative to total muscle wet weight.

Statistical analyses

Unless otherwise stated, data are presented as means $\pm$ SEM. Because no myocyte death occurred in the muscles of control animals, i.e., a zero baseline, non-parametric tests were used to analyze the data relating to myocyte death. Multiple analyses were made using Kruskal-Wallis one-way analysis of variance by ranks and post-hoc analyses were conducted using multiple Mann-Whitney-U tests. Data on muscle protein content and fiber CSA were analyzed using one-way analysis of variance with Tukey (HSD) post-hoc analysis.

Results

At the beginning of the experiment, the average body weight (mean $\pm$ SD) of the animals was 285 ± 7 g and there were no significant differences between the groups. The body weight of control animals infused with saline increased to 311 ± 10 g after 14 days. The infusion of clenbuterol induced significantly ($P < 0.05$) greater increases in body weight than evident in the saline control animals. This became apparent earlier in response to the larger doses, i.e., after 4 d with 1 mg, 6 d with $100 \mu\text{g}$, and 12 d with the infusion of $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of clenbuterol. After infusion with clenbuterol the animal body weights were 309 ± 12 g, 328 ± 6 g, 343 ± 18 g, and 341 ± 6 g for the $1 \mu\text{g}$, $10 \mu\text{g}$, $100 \mu\text{g}$, and $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ groups, respectively.

As expected in growing animals, the wet weights of the heart and skeletal muscles increased significantly ($P < 0.05$) during the 14 d period. There were no differences in the protein concentrations of the muscle homogenates between clenbuterol- and saline-treated animals. Thus, the changes in total protein contents (Table 1) were congruent with the changes in muscle wet weights. When compared to the day 14 saline-infused controls, the protein contents of all muscles had significantly ($P < 0.05$) increased in animals administered doses of $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of clenbuterol or greater (Table 1) whereas infusion of the lowest dose of $1 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of clenbuterol had no significant anabolic effect.

To investigate the changes in muscle protein content, the CSA of myofibers expressing either type I or II MHC was measured (Fig. 1). The average percentage of myofibers expressing type II MHC was 17 % in the soleus, 65 % in the diaphragm, and 94 % in the plantaris and tibialis anterior. These proportions did not alter significantly in any of the muscles after clenbuterol infusion. Clenbuterol-induced anabolism was associated with hypertrophy of myofibers expressing predominantly type II MHC in all skeletal muscles.

Myocyte death was investigated using immunohistochemistry on cryosections of the heart, soleus, diaphragm, plantaris, and tibialis anterior muscles. No myocyte death was detected in the muscles harvested from control animals killed either on day 0 or after infusion with saline. Similarly, no myocyte death was detected in any of the muscles investigated after infusion with low doses of $1 \mu\text{g}$ or $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of clenbuterol. In contrast, infusion of the larger doses ($100 \mu\text{g}$ and 1 mg) of clenbuterol induced myocyte apoptosis (Fig. 2A) and necrosis (Fig. 2B). Myocyte death was detected in the heart, soleus, diaphragm, and plantaris muscles (Table 2). In addition to the overt apoptotic and necrotic myocyte death, small myofibers with centralized nuclei (Fig. 1D; indicative of previous myocyte damage) were also observed (average, 4.7 ± 1 % vs. 0.1 ± 0.01 in the controls; $P < 0.01$) in the plantaris and soleus muscles of animals infused with doses of $100 \mu\text{g}$ or $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of clenbuterol. Discrete regions of the left ventricular subendocardium of these animals had a reduced density of cardiomyocytes (Fig. 2F) and an apparent increase in connective tissue.

Picric sirius red staining verified the increased area fraction of collagen in the hearts of animals administered high doses of clenbuterol (Fig. 2H). When quantified, the fractional area of collagen in hearts from the 1 mg clenbuterol group ($13.3 \pm 0.9 \%$) was significantly ($P < 0.05$) greater than that ($10.1 \pm 0.8 \%$) in the hearts harvested from animals infused with saline only (Fig. 3). No significant changes were observed in the fractional area of collagen in the skeletal muscles.

Discussion

We investigated the dose-dependent myotoxic and hypertrophic effects of controlled infusions of clenbuterol. Significant muscle growth was evident after infusion of doses of $10 \mu\text{g.kg}^{-1}.\text{d}^{-1}$ or greater whereas doses of $100 \mu\text{g.kg}^{-1}.\text{d}^{-1}$ or greater induced muscle growth and myocyte death. The increase in muscle growth induced by the low, non-myotoxic dose of $10 \mu\text{g.kg}^{-1}.\text{d}^{-1}$ of clenbuterol was consistent with an increase in muscle protein content (Table 1) and CSA of the skeletal myofibers (Fig. 1).

Previous work from our laboratory 5, 6 has demonstrated that bolus injection of doses as low as $10 \mu\text{g.kg}^{-1}$ of clenbuterol induces significant myocyte death in the heart and soleus muscle of rats. In response to sustained exposure to an agonist, β -AR desensitization and downregulation occur 2 resulting in tachyphylaxis. However, because of clenbuterol's long plasma half-life and the fact that it accumulates in specific tissue compartments, particularly the heart 35, it is difficult to predict whether chronic administration would be more or less myotoxic than a bolus injection. Recently, we discovered that repeated bi-daily injections of $10 \mu\text{g.kg}^{-1}$ of clenbuterol induced cumulative skeletal myofiber death. However, the incidence of myofiber death measured after each subsequent injection diminished and the myofibers that were lost were later replaced 4. In the current work, only infusion of large doses ($100 \mu\text{g}$ and 1 mg) of clenbuterol induced myocyte death, the incidence of which (average, 0.2%) was less than that observed in our previous work using single injections (i.e., peak values of $\sim 6 \%$), suggesting that tachyphylaxis, possibly due to receptor downregulation 16, is the predominant response. Interestingly, infusion of $100 \mu\text{g}$ or 1 mg of clenbuterol $\text{kg}^{-1}.\text{d}^{-1}$ was able to induce myofiber death in the diaphragm and plantaris muscles whereas these muscles were not damaged by single injections 4. The diaphragm and plantaris muscles have a greater proportion of fast-contracting fibers expressing predominantly type II MHC than the predominantly slow-twitch soleus muscle. Slow-twitch muscles have a greater proportion of β -AR than fast-twitch muscles 20 and accordingly, slow-twitch fibers are more susceptible to the acute damaging effects of β -AR stimulation 1. However, the current finding reveals that chronic β -AR stimulation can induce myofiber death in predominantly fast- as well as slow-twitch muscles. A limitation of the current study is that the time course of myocyte death in response to continuous infusions of clenbuterol was not investigated. From our previous experience using single injections 4, we know that myocyte apoptosis peaks 4 h after and necrosis 12 h after bolus administration of clenbuterol, and also that myocyte death is not detectable after more than 24 h. Thus, the seemingly modest numbers of apoptotic and necrotic myocytes detected after 14 d (Table 2) are indicative of more substantial myocyte loss over this 2-wk period. It was impractical to investigate the time course of myocyte death induced by each dose of clenbuterol throughout the 14-d intervention. However, the observed small myocytes with centralized nuclei in the soleus and plantaris muscles of animals infused with either $100 \mu\text{g}$ or $1 \text{ mg.kg}^{-1}.\text{d}^{-1}$ of clenbuterol (Fig. 2D) supports the suggestion that myocyte death had occurred in these muscles at an earlier point in time. Although the incidence of cardiomyocyte death was not statistically significant after 14 days of infusion, the area fraction of collagen in the heart was significantly ($P < 0.05$) increased (Fig. 3). This increase in myocardial collagen in response to β -agonist administration is consistent with previous reports 13, 16, 29 and may be indicative of reparative fibrosis resulting from cardiomyocyte death 3. The current

findings suggest that many of the previously published investigations that have used large doses of clenbuterol (i.e., greater than $100 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) to induce muscle hypertrophy very probably also induced muscle damage.

Infusion of $10 \mu\text{g}$ of clenbuterol $\text{kg}^{-1} \text{d}^{-1}$ was not myotoxic and induced a significant ($P < 0.05$) increase in the protein content of the heart and slow- and fast-twitch skeletal muscles (Table 1). In agreement with previous studies 12, 23, 31 that used large doses, the hypertrophic affect of clenbuterol was more prominent in myofibers expressing type II MHC (Fig. 1). Furthermore, the magnitude of the anabolic effect was similar to, or greater than, that previously reported in response to larger doses of this agent (Table 3). In the previous literature, clenbuterol-induced muscle growth has commonly been expressed relative to body weight. The normalization of muscle data to body weight may be convenient, because it allows data from animals of varying body weights to be compared, but may not represent good experimental practice. β_2 -AR agonists simultaneously increase muscle mass whilst decreasing adipose tissue mass (i.e., a repartitioning effect), as reviewed elsewhere 39, therefore the amount of muscle growth induced is likely to be exaggerated based on body weight normalisation. In the current work, the initial body weight of all animals was controlled to within $\pm 2.5\%$, and weight-matched day 0 and saline infused control animals were included. Thus, absolute muscle weights (recorded to within $\pm 0.001 \text{ g}$) were used, rather than normalizing muscle weight to body weight (recorded to within $\pm 1 \text{ g}$), thereby increasing the precision of the measurement 1000-fold and negating the influence of clenbuterol on body composition.

Based on the findings of the current work, the dose of $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of clenbuterol can be used as a model of clenbuterol-induced hypertrophy in the absence of myocyte death. This supports the suggestion that this dose is equivalent to the therapeutic dose for humans 24. The hypertrophic potential of this low dose of clenbuterol has been investigated previously 8, 9, 24. Maltin et al. 24 reported that this dose of clenbuterol was able to attenuate the atrophy induced by muscle denervation, but in their study it had no anabolic affect on innervated skeletal muscle or the heart. The apparent discord with the data presented here may possibly be explained by the difference in method of clenbuterol administration. That is, administration of clenbuterol via the animal's drinking water as used by Maltin et al. 24 or via food in other studies introduces uncertainty regarding the actual dose received by each animal, particularly if they are housed in communal cages. In addition, clenbuterol solutions are sensitive to light and oxidation of the agent may occur, resulting in a reduction in the effective dose received by the animal over time. The administration of clenbuterol by subcutaneously implanted osmotic pumps ensures that the dose received by each animal is consistent. It is possible that this stringent control over the administration of clenbuterol and our use of absolute muscle weights and protein contents has enabled us to detect the anabolic effect of this low dose of clenbuterol more precisely.

Using the rat hindlimb-suspension model of disuse, Chen and Alway 8 reported that administration of $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of clenbuterol, via a subcutaneously implanted slow release pellet, did not significantly increase muscle mass. Nevertheless, it did attenuate the loss of peak isometric force produced by the soleus muscle in elderly rats. Unfortunately, Chen and Alway 8 did not include a clenbuterol-only control group that was not subjected to hindlimb disuse, and their muscle weight data were expressed relative to animal body weight. In a later study, the same authors 9 reported that $10 \mu\text{g}$ of clenbuterol also attenuates the loss of fatigue resistance in the soleus muscle, which is concomitant with the disuse in the hindlimb suspension model. This is contrary to the reduction in fatigue resistance 12 and the decrement in exercise performance 13, 22 associated with larger doses (i.e., $\text{mg} \cdot \text{kg}^{-1}$) of this agent that also induce myocyte death.

In conclusion, the current work demonstrates that it is possible to separate the potentially beneficial anabolic effects of clenbuterol from its detrimental myotoxic effects by stringently controlling the dose administered. Furthermore, there is little to be gained by administering larger doses of clenbuterol in order to try and achieve a greater anabolic effect. Rather, the infusion of large doses of the β_2 -agonist is detrimental, inducing myocyte death in the heart and skeletal muscles.

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Glossary

Abbreviations.

Ab	antibody
ATP	adenosine tri-phosphate
AR	adrenergic receptor
CSA	cross-sectional area
EDTA	ethylene diamine tetraacetic acid
MHC	myosin heavy chain

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Fig. 1. Clenbuterol-induced changes in myofiber cross-sectional areas. Cross-sectional area of myofibers expressing predominantly type I (left) or type II (right) MHC isoforms. Data are presented as mean $\pm$ SEM ($n > 6$, in each group). * $P < 0.05$ significantly different from the saline controls.

Fig. 2. Muscle damage induced by continuous infusions of clenbuterol. Myocyte apoptosis (arrow) detected on a cryosection of the plantaris muscle (A; $\times 200$ magnification) and cardiomyocyte necrosis (arrow) detected on a cryosection of the heart (B; $\times 400$ magnification) from animals infused with $100 \mu\text{g.kg}^{-1}.\text{d}^{-1}$ of clenbuterol. Hematoxylin and eosin staining of soleus muscle cryosections ($\times 200$ magnification) from animals infused with saline only (C) or $100 \mu\text{g.kg}^{-1}.\text{d}^{-1}$ of clenbuterol (D); arrows point to small myofibers with centralized nuclei. Hematoxylin and eosin (E and F) or picric sirius red (G and H) staining of cryosections ($\times 200$ magnification) of the left ventricular subendocardium from animals infused with either the saline vehicle (E and G) or 1

mg.kg⁻¹.d⁻¹ clenbuterol (F and H); arrows point to areas of reduced cardiomyocyte density (F) or increased collagen content (H).

Fig. 3.

Clenbuterol-induced changes in myocardial collagen content.

Area fraction of collagen in the left ventricular subendocardium of animals infused with doses of clenbuterol from 1 μ g to 1 mg.kg⁻¹.d⁻¹ or the saline vehicle only. Data are presented as means $\pm$ SEM (n > 6, in each group). *Significantly ($P < 0.05$) different from control animals infused with saline.

Table 1

Protein content (mg) of muscles from control and clenbuterol-treated animals.

	Heart (mg)	Soleus (mg)	Tibialis anterior(mg)	Plantaris(mg)
<u>Controls</u>				
Day 0 (<i>n</i> =6)	125.8 ± 6.3	19.2 ± 2.2	84.5 ± 6.3	38.9 ± 2.2
Day 14 (<i>n</i> =9)	140.6 ± 7.7 *	21.8 ± 1.7 *	104.9 ± 9 *	47.4 ± 4.6 *
<u>Clenbuterol (kg⁻¹.d⁻¹)</u>				
1 µg (<i>n</i> =6)	136.2 ± 6.8	20.2 ± 1.5	101.3 ± 0.9	46.8 ± 1.7
% change	-3.2	-7.4	-4.5	-1.3
10 µg (<i>n</i> =8)	156.9 ± 9.9 *†	24.5 ± 1.9 *†	116.9 ± 8.2 *†	55.7 ± 4.9 *†
% change	+11.6	+12.4	+11.4	+17.5
100 µg (<i>n</i> =6)	165 ± 12.2 *†	24 ± 1 *	128.7 ± 10.7 *†	57.5 ± 5.8 *†
% change	+17.5	+10.1	+22.7	+21.3
1 mg (<i>n</i> =6)	161 ± 8.2 *†	23.3 ± 1 *	128 ± 4.4 *†	61.2 ± 2.3 *†
% change	+14.5	+7.3	+22.1	+29.3

Data are presented as mean ± SD. Percent change represent differences in total protein content (mg), compared with the day 14 saline controls. One-way analysis of variance revealed significant ($P<0.001$) differences between the groups (clenbuterol or saline infused) for the heart, soleus, tibialis anterior and plantaris.

* $P<0.05$, significantly greater than day 0 control.

† $P<0.05$, significantly different from the day 14 saline controls (Tukey HSD post-hoc analyses).

Table 2

Myocyte death induced by continuous infusions of clenbuterol.

Dose $\text{kg}^{-1} \cdot \text{d}^{-1}$	Muscle	% Apoptosis	% Necrosis
100 μg	Heart	0.02 ± 0.01	0.04 ± 0.03
	Soleus	$0.2 \pm 0.1^*$	0.0
	Plantaris	0.08 ± 0.02	0.01 ± 0.05
	Diaphragm	$0.15 \pm 0.09^*$	0.0
1.0 mg	Heart	0.04 ± 0.02	0.05 ± 0.02
	Soleus	0.0	0.0
	Plantaris	$0.21 \pm 0.05^*$	$0.3 \pm 0.05^*$
	Diaphragm	0.14 ± 0.1	0.0

Data are presented as means $\pm$ SEM ($n > 6$, in each group) and represent percent area in the heart or percent number of fibers in the skeletal muscles that stained positively for myocyte death.

* Significantly ($P < 0.05$) different from the baseline of zero myocyte death in the muscles of control animals harvested on day 0 or animals infused with saline only or doses of $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of clenbuterol or less.

Table 3

Muscle growth reported in the literature after controlled administration of clenbuterol to laboratory rats.

Dose	Duration	% increase in muscle mass					Reference number	
		Sol	Gas	PI	EDL	TA	Heart	
0.25*	7	15	22	--	--	--	6	23
0.25*	56	11	--	--	23	--	--	31
1.0*	14	17	17	17	19	15	10	38
1.0*	16	--	13	--	--	--	3	14
1.0*	21	--	5	--	--	--	--	32
1.0*	42	17	--	--	--	--	--	11
2.0*	14	6	18	15	--	--	--	12
2.0*	105	17	--	--	9	--	--	22
3.0	9	--	12	--	--	11	--	18
0.01	14	12	--	18	--	11	12	Table 1

Doses of clenbuterol ($\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) were given over different durations (days) as subcutaneous injections* or continuous infusion via subcutaneously implanted osmotic pump. Increases in the wet weight of the soleus (Sol), gastrocnemius (Gas), plantaris (PI), extensor digitorum longus (EDL), tibialis anterior (TA) and heart are expressed (% increase) relative to saline controls. Dashes (--) indicate that the muscle mass was not measured. Data from the current work (Table 1) using the non-myotoxic dose of $10\text{ }\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ clenbuterol have been included for comparison.