Journal of Sports Medicine & Doping Studies
ISSN: 2161-0673
Douillard, J Sport Medic Doping Studie 2011, S1
DOI: 10.4172/2161-0673.S1-001
Review Article Open Access

Skeletal and Cardiac Muscle Ergogenics and Side Effects of Clenbuterol Treatment

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Abstract

Well known, well detected and still used by athletes, clenbuterol is one of the β_2-agonists which has no authorization for therapeutic use, contrarily to salbutamol, salmeterol and formoterol in the 2012 World Anti-Doping Agency list. However, clenbuterol is still detected in athletes' antidoping test samples. Its ability to induce muscle hypertrophy but also its strong lipolytic action and the absence of androgenic effects have made it a prized substances by athletes, specially females, without scruples whose performance requires significant muscle strength. Like the effects of clenbuterol on the heart, the effects of clenbuterol on skeletal muscle are dependent on the doses used and duration of the treatment. If there is a consensus concerning the clenbuterol action on the phenotypic conversion from slow to fast type fibers and on the hypertrophy, there is, to our knowledge, no consensus concerning the effects of clenbuterol on the slow type fibers and slow profile muscles. There is also no consensus concerning the clenbuterol effects on performance.

We will shortly reviewing the known operating mode, side and benefits effects of short and long term β-agonists, and specially clenbuterol, treatment on mammals.

Introduction

Among the various pharmacological doping substances, the β_2-agonists were first used for their bronchodilatory property by smooth muscle relaxation. According to the World Anti-Doping Agency (WADA) the use of all β_2-agonists and their isomers D and L is banned in sport. An authorization for therapeutic use may be granted for 3 β_2-agonists, salbutamol, salmeterol and formoterol in an inhaled form. However, clenbuterol is placed on the banned list in the class of anabolic agents and that of β-agonists. Its ability to induce muscle hypertrophy but also its strong lipolytic action and the absence of androgenic effects have made it a prized substances by athletes, specially females, without scruples whose performance requires significant muscle strength.

The importance of the signaling pathway of β_2-adrenoceptors in the heart is well known today, however, only recently have we begun to understand and take into consideration the importance of this pathway in the skeletal muscle. Yet since the early 1980s, numerous studies have demonstrated the effect of β_2-adrenoceptors stimulation on the growth of skeletal muscle [1-15]. Although originally used to treat bronchospasm, it became apparent that some β-agonist could increase skeletal muscle mass and decrease body fat. These side effects have proven to be of interest to the livestock industry which has tried to increase the intake of animal weight and to improve meat quality, but a series of food poisoning across the Europe have led to a ban on the use of clenbuterol in animal husbandry in 1996.

The effects of β-agonists on skeletal muscle and heart, allowed us to identify potential therapeutic applications in conditions of muscle loss, attempting to mitigate or reverse the muscle wasting and weakness associated, but also trying to improve muscle growth after injury [1,3,16,17]. However, side effects were observed on heart and have limited the application of β-agonists and their therapeutic potential [18,19]. Yet these β-agonists have been repeatedly used for doping [20,21], because chronic treatment leads to a phenotypic conversion to a higher fast skeletal muscle profile and a hypertrophy of muscle fibers without dependent androgenic effects.

Operating Mode

Adrenergic receptors belong to the family of guanine nucleotide binding G protein-coupled receptor (GPCR) implicated in the regulation of cardiovascular, respiratory, metabolic and reproductive functions. The β-adrenoceptors belong to the subfamily of rhodopsin receptors which also include dopaminergic, adenosine and histamine receptors [22,23]. These receptors are coupled to a G protein composed of three subunits (α, β, γ). The structure of the GPCRs is composed of seven transmembrane α -helices forming three extracellular loops, including the NH2 terminus and three intracellular loops including the COOH terminus [24,25]. There are three subtypes of β-adrenoceptors, β_1, β_2 and β_3 [26-29], which possess 65-70% homology [30].

Anabolic pathway

In skeletal muscle, the proportion of β_2- adrenoceptors is about 90%, that of β_1- adrenoceptors ranged from 7 to 10% and β_3-adrenoceptors are located in fat cells and in cardiac muscle [31]. In addition, β- adrenoceptors have a higher density in slow muscles like Soleus than in fast muscles such as Extensor Digitorum Longus (EDL) [32]. However, the functional significance of this difference in density is not well understood, in fact, the response to treatment with β-agonists appears to be greater in fast muscles than in slow muscles [33,34]. This may be partly explained by the down-regulation of receptors following prolonged treatment [14].

The β_2-adrenoceptors bind to and $G\alpha_s$ and $G\alpha_i$ proteins [35-37]. The protein $G\alpha_i$ is essential in the spatial localization of $G\alpha_s$ and in the ensuing cyclic Adenosine MonoPhosphate (cAMP) response [38,39]. This cAMP dependent pathway is one of the pathways responsible for hypertrophy induced by β_2-adrenergic stimulation in

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Received November 03, 2011; **Accepted** December 24, 2011; **Published** December 29, 2011

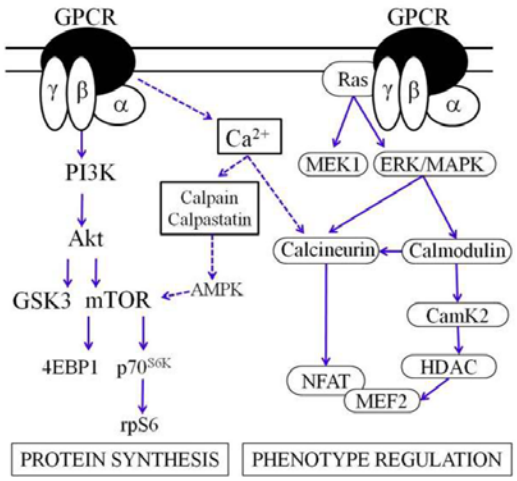
Citation: Douillard A (2011) Skeletal and Cardiac Muscle Ergogenics and Side Effects of Clenbuterol Treatment. J Sport Medic Doping Studie S1:001. doi:10.4172/2161-0673.S1-001

skeletal muscle. In addition, the Gβγ heterodimer is able to initiate a response independent of Gα subunit after β_2-adrenergic stimulation [40]. Indeed, the Gβγ dimer can activate the signaling pathway of Phosphatidylinositol 3-kinases (PI3K) [41]. PI3K is an essential protein involved in the activation of Protein Kinase B (Akt) through PIP2/PIP3 who creates two lipid binding sites to Protein Kinase B. Akt could then be phosphorylated and activated by 3'-Phosphoinositide-Dependant protein Kinase 1 (PDK). Akt is known to activate downstream effectors like Forkhead box (FoxO), Mammalian Target Of Rapamycin (mTOR), 4E binding protein 1 (4EBP1) or ribosomal protein S6 (rpS6), well known to be involved in protein synthesis, gene transcription or cell proliferation [42-44] (Figure 1).

Catabolic pathway

Some studies in the early 90's have recorded variations in the calpain system induced by clenbuterol treatment. Calpains are Ca^{2+}-dependent cysteine proteases that constitute a large and diverse family. Skeletal muscle fibers contain ubiquitous calpain 1 and 2 but also calpain 3 (p94) which has, since recently, been described as a muscle-specific calpain [45-47]. Calpastatin is the endogenous protein that specifically inhibits the proteolytic activity of calpains including both calpains 1 and 2 [48]. However, the activity of ubiquitous calpains depends on many factors other than calpastatin, such as Ca^{2+} concentration, autolysis, intracellular localization and, although not yet clearly defined, by phosphorylation [49]. The precise roles and normal regulation of calpains in skeletal muscle are currently unclear, although they are likely to be involved in cytoskeleton organization, the cell cycle and apoptosis [48]. Calpains can degrade cytoskeletal and myofibrillar proteins [50,51] but are mostly involved in limited proteolysis of some specific target proteins [48]. Several studies reported that calpains are involved in skeletal muscle remodelling and atrophy.

In skeletal muscle, a decrease in calpain 1 activity along with an increased calpain 2 and calpastatin activities/expression follow chronic high doses of clenbuterol [52,53]. Recently, we show that 21 days of clenbuterol treatment induced an early activation of the skeletal muscle calpain system as judged by the increased calpains' activity and calpain 2 autolysis that occur both in fast and slow muscles 12h after the first injection [5]. The catabolic pathway of ubiquitin proteasome seems to be also involved in hypertrophic process under clenbuterol action. Indeed, Yimlamai et al. has shown that rats treated with clenbuterol had a reduced ubiquitin-proteasome activity in an Insulin Growth Factor (IGF-1) independent manner [54]. Hence, there is, to our knowledge, no consensus concerning the importance of the catabolic pathway in the clenbuterol induce muscle remodelling.



Arrows represent action (activation or inhibition) on downstream effectors.

Figure 1: Signaling pathways probably involved in clenbuterol induced muscle hypertrophy and phenotypical shift.

Main Effects and Applications

The primary effect of clenbuterol is a relaxation of smooth muscle causing bronchodilation. However, the use of β-agonists in the breeding industry has highlighted many side effects. Following treatment with clenbuterol, the main findings reported are the increase in lean body mass, decreased fat mass and thus increasing the ratio lean mass/fat mass. There is also a phenotypic conversion of slow fibers to fast fibers but also hypertrophy of muscle fibers that is dependent on muscle studied and the type of fiber. We observe, finally, abnormalities of calcium homeostasis during treatment with β-adrenergic including reducing sarcoplasmic reticulum Ca^{2+} loading in the EDL and Soleus muscles induced by an increase in the liability release [55,56].

Increase in mass ratio lean / fat mass

Studies in the breeding industry showed that after a treatment with a β-agonist an increase in lean mass concomitant with a decrease in body fat of treated animals compared to control animals was observed [57-67]. Several studies in humans and animals, showed a strong lipolytic effect resulting especially from the thermogenic properties of the β-agonist [65,68-75]. Adipose tissue is a major site for both thermogenesis and of course for the storage of fat. The β-agonists, including clenbuterol, act on receptors of this tissue to increase lipolysis [76]. Precisely on small animal and specifically with clenbuterol, there is a 36 g increase in mass of Wistar rats after treatment for one week (250 $\mu g.kg^{-1}.d^{-1}$) while rats receiving placebo treatment have had an increase of 18 g [11]. Another study reported that treatment (1 $mg.kg^{-1}d^{-1}$) for 15 days increases by 9 % the weight of the rats while reducing (4 %) the food intake [77]. Furthermore, treatment (1.5 $mg.kg^{-1}d^{-1}$) for 3 weeks increases skeletal muscle mass, in an age dependent manner: the more the rats are young, the more the skeletal muscle mass increase is important. Hence, for 3 months old rats, the skeletal muscle mass compared to the total mass represents 22 % of control rats and 39 % of clenbuterol treated rats, whereas the same treatment for 3 weeks in for 23 months old rats, the skeletal muscle mass compared to the total mass represent 22 % in the control rats and 25 % of the clenbuterol treated rats [3].

Cardiac muscle

Effects when taking acute: Various studies have examined the dose-dependent responses in the heart following acute treatment with clenbuterol. It should be noted studies from Burniston et al. [78] which suggested a deleterious effect of clenbuterol on cardiomyocytes at doses between 0.01 $mg.kg^{-1}$ and 5 $mg.kg^{-1}$. Indeed, at these doses, their studies show an onset of necrosis of cardiac muscle fibers in endocardial left ventricle. It is important to note here the difference between necrosis and apoptosis. Indeed, there are two phenomena of cell death. On the one hand, necrosis is the premature cell death; it can be caused by external factors such as infection or administration of toxins (poisonous bite of an animal for example). On the other hand, apoptosis is the programmed cell death. It is the process by which cells trigger their destruction in response to a signal [79]. Unlike necrosis, apoptosis does not induce inflammation and cell membranes are not destroyed. At an injection of clenbuterol at a dose of 0.01 $mg.kg^{-1}$, necrosis reaches about

4 % fibers [80] and nearly 8 % of the fibers with the administration of larger doses (5 $mg.kg^{-1}$). The same study shows that the peak of necrosis of cardiac fibers is reached between 12 to 15 hours after administration of clenbuterol. Another more recent study [81] shows a time effect and dose-dependent apoptotic-necrotic reactions. Indeed, apoptotic phenomena, demonstrated immunohistochemically by an antibody directed against a marker of apoptosis, the caspase 3, appear less than an hour after the subcutaneous injection of clenbuterol at doses between 1 $mg.kg^{-1}$ and 5 $mg.kg^{-1}$. This apoptotic phenomenon reached a peak 4 hours after injection. However, the number of apoptotic cells is limited and does not exceed 0.8 % of the total area studied. In the same study, the phenomena of necrosis, revealed by an antibody against myosin and injected intraperitoneally one hour before the injection of clenbuterol, appear 3-4 hours after injection at doses between 100 $\mu g.kg^{-1}$ and 5 $mg.kg^{-1}$. This phenomenon reached its peak of necrosis 12 hours after injection as shown in the first study. These results suggest a rapid implementation of processes for the destruction of heart muscle cells.

Effects of chronic intake: Many studies have investigated the possible effect of chronic treatment for several days on the heart muscle. In a first study, Emery et al. [6] observed no effect of clenbuterol (2 $mg.kg.^{-1}d^{-1}$) after one week of treatment on the mass of the heart muscle. Similarly, according to a study by MacLennan and Edwards, one week of treatment with clenbuterol did not significantly increase the mass of the heart in rats [11]. However, in this study, doses of clenbuterol injected remained low (250 $\mu g.kg^{-1}.d^{-1}$) compared to other studies. Indeed, Choo et al. observed in their study, after 4 days of treatment (4 $mg.kg.^{-1}d^{-1}$), an increase of 12 % of the mass of the heart [4]. Equivalently, treatment of 2 $mg.kg^{-1}d^{-1}$ causes an increase of 18 to 20 % of cardiac mass after 2 and 5 weeks of treatment [82]. Clenbuterol is an agent that can induce cardiac hypertrophy, but it seems necessary to apply, to observe that hypertrophy, a treatment with high doses or long treatment [14,33,83-85]. However, the prolonged administration appears to have toxic effects on the heart [19,86]. Duncan et al. [87] have particularly highlighted the infiltration of collagen in the heart of rats treated with clenbuterol. This appearance of collagen could be related with phenomenon such as apoptosis, necrosis and inflammation described by Burniston that may precede the infiltration of collagen [80]. High doses of clenbuterol (2 $mg.kg.^{-1}d^{-1}$) administered for several months induced a strong left ventricular hypertrophy, but also infiltration of collagen and mechanical damage such as reducing the pressure in the left ventricle [87,88]. However, β-As have beneficial effects on the heart as they have been used to treat patients after a heart attack to limit or counteractatrophy [82,89,90].

The effects of β-agonists on the heart are dependent on dose and duration of treatment. Thus, to observe cardiac hypertrophy treated with high dose over several weeks seems necessary. However, the deleterious effects of high doses taken in acute or chronic treatment (necrosis, infiltration of collagen) limit the therapeutic uses of these β-agonists.

Skeletal muscle

As we have seen previously, clenbuterol induced an increase in skeletal muscle mass during treatment. Two phenomena are responsible for this increase in mass, the phenotypic conversion of muscle fibers towards a faster profile and hypertrophy.

Effects of clenbuterol on the phenotypic conversion: During prolonged treatment with clenbuterol, there is a phenotypic conversion to a faster profile of skeletal muscle. Chronic administration of clenbuterol to rats or mice leads to a transition from slow (type I) to fast (type II) muscle fibers [1,57,91-95]. In addition, the phenotypic conversion is also observed among the fast fibers, from type IIa fibers to type IIx fibers or fiber type IIb [93]. This transition towards a more glycolytic profile is not only metabolic (glycolytic or oxidative) but also structural with changes in the MHC isoform composition and therefore in the contractile properties of the muscle [96]. These phenotypic changes usually occur during muscle development [97], following a protocol of electrical stimulation at high frequencies [98] during denervation or hormonal changes [99], while reducing load during a simulated loss of gravity during muscle regeneration [7] and in a more limited manner following a training protocol [100,101].

The mechanisms that control the phenotype of slow muscle fibers were studied and some signaling pathways have been described, including many factors such as calcineurin and Nuclear Factor of Activated T-cells (NFAT) [102,103], Ca^{2+}/calmodulin dependant kinase (CamK) [104], the Peroxisome proliferator-activated receptor Gamma Coactivator 1 (PGC1α pathway) [105] and that of Peroxisome Proliferator-Activated Receptor δ (PPAR-δ) [106] or Ras [107]. However, the mechanisms controlling the expression of a fast muscle phenotype are less known. A work by Grifone et al. [108] suggest that the binding of Six1 to the cofactor Eya1 exerts a transcriptional regulation of the expression of fast MHC. The complex Six1/Eya1 would bind to MEF3 to induce expression of MHC proteins quickly. Indeed, when cotransfecting plasmids of Six1 and Eya1 by electroporation in the soleus muscle of mice, it was observed a phenotypic conversion of slow fibers I and IIa to faster IIb fibers. Recent data from Richard & Maire, shows that absence of Six1 and Six4 leads to the development of dorsal myofibers lacking expression of fast-type muscle genes indicating a probable implication of Six1 and Six4 in the regulation of fast-twitch MHC [109]. However, even if the administration of clenbuterol produces a similar effect, no study has, for now, highlighted an influence of the clenbuterol on the expression levels of Six1 and Eya1 in the skeletal muscle.

Effects of clenbuterol on hypertrophy: As discussed above, chronic treatment with clenbuterol leads to increased skeletal muscle mass. This increase in mass is treated as a pure muscle cell hypertrophy [110] because hyperplasia is not associated with increased protein [93]. Other β_2-agonists such as cimaterol, salbutamol or isoproterenol induce hypertrophy, but when administered at high doses, clenbuterol is one of the most effective agents for inducing hypertrophy. The ability of clenbuterol to induce muscle protein synthesis [4,6,12,65,66,111,112] and reduce protein degradation [12,65,93,113-115] was the basis of demonstration of its anabolic power. However, there is no consensus on a preferential mechanism responsible for this clenbuterol induced hypertrophy. A more recent study highlights the ability of clenbuterol to activate the signaling pathway Akt/mTOR highlighting the fact that clenbuterol is responsible for both a decrease in protein degradation and increased protein synthesis [116]. On the other hand, the study of Shi et al. suggests that the Mitogen-Activated Protein Kinase (MAPK) signaling pathway is involved in hypertrophy induced by clenbuterol [117]. The same study shows that Extracellular signal-Regulated Kinase (ERK) is differently regulated between the fast muscles and the slow muscles. Indeed, following treatment with clenbuterol, ERK activity in the soleus is increased by 39 % whereas in the Tibialis Anterior (TA) and the Gastrocnemius, there was an increase of 2.3 and 2.5 times the control levels of the ERK activity.

It should be noted that the effects of clenbuterol are different depending on the species and it seems that the effects in humans are

less pronounced than in the farmed species. Moreover, the effects within a species vary depending on the tissue, mainly because of the density and distribution of different subtypes of receptors for a given tissue and species [118,119].

The administration of β_2-As leads to hypertrophy of type I fibers [57,94,120] and type II fibers [15,64], but some studies show a similar increase in the size section of different types of fibers [121,122]. Here again, consensus does not exist. However, the disparity in species and age of animals at the beginning of treatment, the β-agonist used and the doses, route of administration, frequency and duration of treatment does not favor the establishment of a consensus.

Effects of clenbuterol on performance: Spann and Winter showed that low doses of clenbuterol don't improve performances [123]. Contrary to them, studies reported that large doses of clenbuterol (1 $mg.kg.^{-1}d^{-1}$/ 2$mg.kg.^{-1}d^{-1}$) could have deleterious effects on rats' performances. Indeed, mice treated with clenbuterol for 8 weeks (1.6 $mg.kg.^{-1}d^{-1}$) and submitted to interval training showed a reduction in total work performance (-25 %) at a run-to-exhaustion treadmill test [124]. Similarly, a treatment period of 14 weeks (2 $mg.kg.^{-1}d^{-1}$) with clenbuterol induced a 50 % decrease in swimming time to exhaustion a 57 % decrease of voluntary running time and a 43 % decrease of speed running [87]. These data suggests that a long clenbuterol treatment could decrease performances by earlier exhaustion.

According to Duncan, the limitations of oxidative capacity induced by β_2-agonists, the decrease in blood flow and cardiac muscle alterations structure are responsible for this decrease in performance [87,124]. Localized collagen infiltration in the left ventricular and increase cardiac mass could have contributed to the overall decrease in exercise training performance. Moreover, as shown previously, clenbuterol is responsible for phenotypic conversion from slow to fast skeletal muscle fibers. This conversion and therefore the greater proportion of fast fibers induces a greater velocity of shortening/contraction [15,125] which could make the muscles less resistant to fatigue [125]. Torgan and colleagues reported that the metabolic phenotype is also modified by clenbuterol treatment in rats. In this study, clenbuterol was responsible for a reduction of muscle oxidative potential by reducing the Citrate Synthase (CS) activity in fast muscle (plantaris, white gastrocnemmius) and this decrease could be minimized by endurance training [126]. Mounier et al. also reported in EDL that strength training seemed to counteract, to some extent, the molecular modifications induced by chronic clenbuterol administration [127]. According to this study, a 2 $mg.kg.^{-1}d^{-1}$ clenbuterol treatment improved activities of PhosphoGlycerate Kinase (PGK) and enolase but decreased PhosphoFructoKinase (PFK) and CS activity. Thus, the activity of some oxidative enzymes is decreased following treatment with clenbuterol whereas the activity of glycolytic enzymes is improved by clenbuterol [126,128-130].

However, isometric force from trained rats treated with clenbuterol (8 weeks to 2 $mg.kg.^{-1}d^{-1}$) is increased compared to trained rats who received no additional treatment [131]. Moreover, clenbuterol is able to induce an increase in the maximum tension evoked without any muscle hypertrophy [15]. Although, clenbuterol induced hypertrophy strength gain relative to muscle mass is no more significant. Indeed, the absolute maximum force is enhanced by clenbuterol while the force relative to muscle mass is unchanged [10,33,125].

Conclusion

Like the effects of clenbuterol on the heart, the effects of clenbuterol on skeletal muscle are dependent on the doses used and duration of the treatment. If the consensus is established on the action of clenbuterol on the phenotypic conversion from slow to fast type fibers and on the hypertrophy, all studies do not meet on the effects of clenbuterol on the slow type fibers and muscles with a slow profile. Thus, it seems clear that the phenotypic conversion occurs in all types of muscle from slow fibers to fast fibers, the speed and the intensity of the shift are not found in identical form in all studies. Similarly, if hypertrophy of fast fibers in all types of muscles is generally found, hypertrophy of type I fibers is one more time highly dependent on the dose used, the duration of the treatment and the type of muscle studied.

Regarding the performance, clenbuterol does not seem so interesting for athletes. As expected, if the effects on endurance performance are negative, the effects on strength or power performance are not necessarily beneficial. Thus, if the maximum speed limit is decreased, the force appears to be enhanced when the hypertrophy is not yet present. When hypertrophy is present, the absolute strength is increased and the relative strength of muscle weight is on her diminished. More work is needed to understand the mechanisms of clenbuterol action and fight more efficiently doping.

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This article was originally published in a special issue, **Muscle Hypertrophy** handled by Editor(s). Dr. Jennifer A. Bunn, Campbell University, USA