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Original Article

Histomorphometrical Evaluation of Mice Testicular Tissue Following Short and Long-Term Administration of Clenbuterol

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Abstract

The aim of this study was to investigate the effects of long-term administration of clenbuterol on the histomorphometry and histopathology of testicular tissue in mice. 30 male NMRI mice were divided into three groups including the control group that received (0.1 ml of normal saline) the second group, (2.5 mg /kg clenbuterol) and the third group (8 mg/kg clenbuterol) that received for 30 consecutive days. At the days 3 and 30 from the beginning of the study, five mice were randomly sacrificed from each experimental group. The testicular tissue was removed from the right testis and fixed in the *Bouin's* solution for histomorphometric and pathological examinations. Degenerative lesions including distortion of the germ cells, reducing their number, and presence of vacuoles within the seminiferous epithelium were demonstrated in the clenbuterol treated groups. At the present study, administration of clenbuterol decreased spermatogenesis parameters including meiosis index, Johansen score, spermatogenesis percentage, seminiferous tubules diameter and epithelium height of seminiferous tubules both in the day 3 in low and high dose of clenbuterol with more severity in high dose. On the 30th day, testicular degenerative changes due to clenbuterol were more in the low dose of rather than high dose. According to the obtained results, it can be concluded that administration of clenbuterol may has potentially negative affect on the sperm production and fertility in male mice.

Keywords: Beta-2 agonist; Testes; Infertility; Degeneration; Seminiferous tubules; Spermatogenesis.

1. Introduction

Clenbuterol [4-amino-alpha-methyl-3, 5-dichlorobenzyl alcohol hydrochloride], a long-acting beta-2 agonist is used as a potent repartitioning agent to increase lean muscle mass in livestock but its use is illegal from FDA instruction [1]. In human, clenbuterol uses is for dilating pulmonary airways by smooth muscle relaxation and decreasing mucus secretion from pulmonary tract epithelium in patients with asthma [2]. Clenbutrol is the most effective bronchodilator that is approved in European

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union, USA and some other countries. Medication is also common in horses with chronic respiratory tract stenosis (COPD) [3]. Additionally, clenbutrol has anabolic properties and its effect for muscle accumulation in sheep, broilers, cows and horses is well known [4]. Clenbutrol decreases adipose tissue as the muscle mass increases [5,6]. It has shown that anabolic effects of clenbutrol are through binding to beta 2-adrenergic receptors on the muscles and motivation of related intracellular signaling pathways [7,8]. Protein anabolic effect of clenbuterol has led to researchers made trials to evaluate efficiency of drug for treatment of various diseases affecting muscle mass or weakness in human [8].

Clenbuterol has side effects on various organs. Prolonged use of this drug decreases aerobic exercise performance in horses, rats and mice [9, 10, 11], leads to cardiac hypertrophy, myocardial fibrosis and cardiac arrest in rats [10]. The administration of clenbutrol at growth promoting doses causes acute toxic effects including muscle tremor, tachycardia, palpitations and nervousness [12, 13]. It is not clear that if beta 2-adrenergic drugs, especially when used for long-term bodybuilding exercise have harmful effects on the reproductive organs in males. Some of the previous studies designed to respond to this question. Clenbutrol receptors have been found in testicular tissue on the Sertoli, Leydig, and germ cells [14, 15]. Degenerative changes were seen following long term administration of clenbutrol in the seminiferous tubules [16]. Clenbuterol can also affect spermatogenesis by binding to its receptors at the germ cell and Sertoli cell surface and induces changes in the cellular organelles structure and function [14, 17]. At the present study, we tried to evaluate effect of long-term oral consumption of clenbutrol on testicular tissue in mice.

2. Materials and Methods

2.1. Animals

In the present study, 30 adult male NMRI mice were purchased from the Kerman Medical University, Kerman, Iran. These animals had 6-8 weeks old and weighting 35-25 g. For adaptation to the laboratory environment, animals were housed in a propylene cage with mesh lid for one week in the Laboratory Animal House ofthe Faculty of Veterinary Medicine of Shahid Bahonar University of Kerman and accessed freely to water and food (pellet form, Javaneh Khorasan Co., Iran) during the study. Storage conditions were set to 12 hours of light and 12 hours of darkness and 22±2°. All investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals published by the National Institutes of Health. The study was approved by ethics committee of Shahid Bahonar University of Kerman (ethical approval No.IR.UK.VetMed.Rec. 1398.013).

2.2. Study design

Male mice were randomly divided to the control and two treatment groups (n=10 in each group). The first treatment group was received clenbuterol at a dose of 2.5 mg/kg in 0.1 cc once a day by gavage (clen-low dose group), the second treatment group was received clenbuterol at a dose of 8 mg/kg in 0.1 cc once a day by gavage (clen-high dose). The control animals received normal saline using the same volume and similar method. The amount of oral

administration of the clenbuterol was chosen in the clen-low dose group based on NOEL (no-observed-adverse-effect level) dose and in clen-high dose group based on approximately one tenth the LD50 dose of clenbuterol in mice [18]. Clenbutrol was purchased from Sigma-Aldrich Company. Five mice were sacrificed at 3 and 30 days following treatment from each experimental group and their right testes were removed for histomorphometrich and histopathological assessments.

2.3. Sample assessments

Each testicular specimen was fixed in Bouin's solution, embedded in paraffin wax, sectioned in 5 μm thicknesses, stained with hematoxylin and eosin (H&E) and evaluated with a light microscope blindly by a pathologist (Nikon, Digital Sight DS-Fi2, Japan). Testicular function evaluation was done based on the previous works [19] and spermatogenesis expressed as Johnsen's score and percent of active spermatogenesis in the seminiferous tubules (number of seminiferous sections with spermatozoa inside them). Miotic index is another parameter for estimation of cell death during meiosis. Miotic index is characterized with the number of round spermatids per spermatocyte undergoing meiosis [20]. For morphometrical assays, diameter and epithelial height in 10 smallest and roundest seminiferous tubules were investigated in each sample.

2.4. Statistical analysis

Our data were analysis by SPSS17.0 (SPSS Inc., Chicago, IL, USA) software. Levene static test was used to assessment of data homogeneity. Comparison between the experimental groups was done using one-way analysis of variance (one-way ANOVA) followed by the least significant difference test (LSD) for multiple comparisons when the variances were homogenous, otherwise Tamhane's test was used as *post hoc*. Values were expressed as mean ± SD. $P \leq 0.05$ was considered as the significant level.

3. Results and Discussion

Clenbuterol is belong to the beta 2-adrenergic drugs, has been used for many years to increase muscle mass in animal industry as well as human bodybuilding because of its androgenic properties [21]. The adverse effects of non-therapeutic use of clenbuterol have been observed in various tissues [22, 23]. Reproductive tissues including the uterus, ovary [24, 25] and testes [16] were considered in order to investigate the side effects of clenbuterol. In the present study, clenbuterol reduced all testicular functions indices including meiosis index, Johnson rating, spermatogenesis percentage, diameter and epithelium height of seminiferous tubules on day 3 and 30 after the onset of treatment. To the best of our knowledge, there is no study that describes testicular morphometrical indices for the use of clenbuterol in mice.

In the control group on day 3 and 30, testis had intact seminiferous tubules with different stages of germ cells (spermatogonia, primary spermatocyte, and secondary spermatocyte) that normally arranged (**Fig.1**).

On 3 days after clenbuterol consumption, the clen-low dose group showed moderate degenerative lesions, including disruption of the germ cells arrangement, reduction in their

number, and vacant spaces within the lining epithelium of the seminiferous tubules (**Fig. 2**).

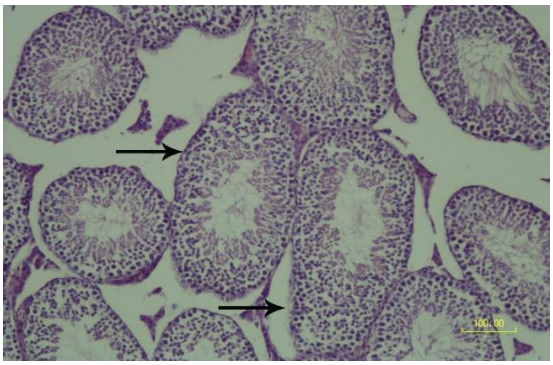

Figure 1. Control group. Photomicrograph showing normal seminiferous tubules morphology (arrows) (H&E staining. Bar=100 µm).

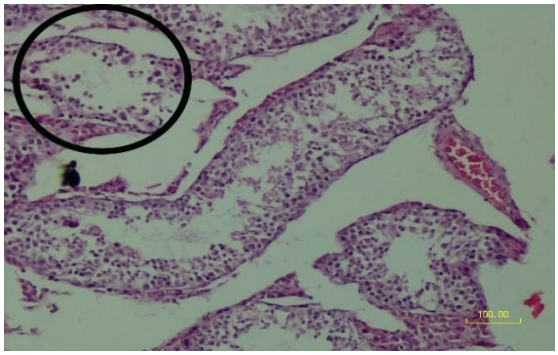

Figure 2. Clenbuterol treated group with a dose of 2.5 mg/kg (Clen low dose group) on 3rd day. Photomicrograph showing degenerative changes. The order of germ cells has been disrupted and their numbers decreased (in the circle) (H&E staining. Bar=100 µm).

In the clen-high dose group, more severe degenerative lesions than in the clen-low dose group at the same day. The seminiferous tubules were smaller and folded, with obvious decrease in germ cells and creating multiple gaps in the tubules lining epithelium (**Fig. 3**). In the Clen low dose group on day 30, degenerative changes slightly were alleviated. In compared with 3th day at the same dose, signs of regeneration in the seminiferous epithelial tissue were seen. In the Clen high dose group on day 30, the seminiferous tubules were markedly regenerated in comparison to the same dose at day 3, and many seminiferous tubules showed hyperplastic and high cellular epithelial tissue (**Fig. 4**).

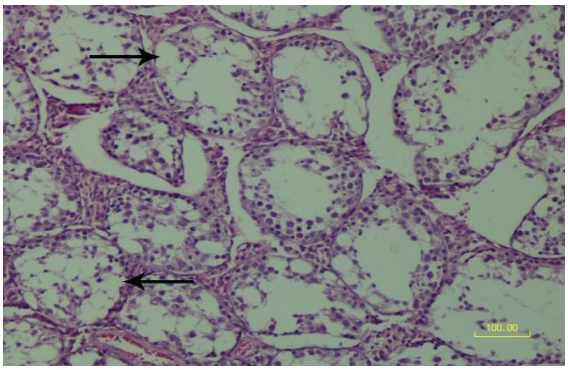

Figure 3. Clenbuterol treated group with a dose of 8 mg/kg (Clen High dose group) on 3rd day. In this image, an obvious decrease in the number of germ cells and empty spaces in the lining tissue are evident (arrows) (H&E staining. Bar=100 µm).

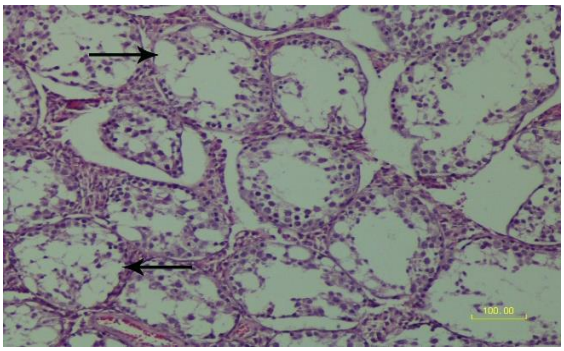

Figure 4. Clenbuterol treated group with a dose of 8 mg/kg (Clen High dose group) on 30th day. An increase in germ cells is seen inside seminiferous tubules, indicating the process of regeneration of tissue in this group (arrows) (H&E staining. Bar=100 µm).

The average percentages of spermatogenesis are listed in **Table 1.** Clenbuterol consumption significantly reduced spermatogenesis in all treated groups on day 3 and day 30 compared to control group ($P \leq 0.05$). At day 3, the rate of reduction in spermatogenesis was significantly higher in the Clen high dose than in the Clen low dose group ($P \leq 0.05$). At day 30, the percentage of spermatogenesis in the Clen high dose was significantly higher than the Clen low dose group ($P \leq 0.05$). The percentage of spermatogenesis in the Clen low dose group on day 30 compared to day 3 showed a significant decrease, whereas in

the Clen high dose group, the percentage of spermatogenesis was significantly higher on day 30 than day 3. This pattern of changes was also in the Johnsen score and miotic index, except that there was no significant difference with the Johnsen score in the low dose group on day 30 compared to day 3 (**Tables 2 and 3**).

Table 1: Mean±SD percentage of spermatogenesis in testes of rats at 3 and 30 days after clenbuterol administration.

Days after clenbuterol administration					
		3rd days		30th days	
Groups	Control	Clen Low*	*Clen High	Clen Low	Clen High
Spermatogenesis percentage	80.60±4.39[a]	56.75±3.32[b]	29.20±16.27[c]	35.80±9.58[c]	56.50±5.17[b]

*Clen Low: Clenbuterol was gavaged with the dose of 2.5 mg/kg/day
*Clen high: Clenbuterol was gavaged with the dose of 8 mg/kg/day
[a,b,c,d]different superscript alphabets show significant difference between experimental groups (P≤0.05)

Table 2: Mean±SD of Johnsen's score in testes of rats at 3 and 30 days after clenbuterol administration.

Days after clenbuterol administration					
		3rd days		30th days	
Groups	Control	Clen low*	*Clen high	Clen low	Clen high
Johnsen's score	7.86±0.28[a]	5.05±0.28[b]	3.47±0.49[c]	5.16±0.40[b]	5.92±0.42[d]

*Clen Low: Clenbuterol was gavaged with the dose of 2.5 mg/kg/day
*Clen high: Clenbuterol was gavaged with the dose of 8 mg/kg/day
a,b,c,ddifferent superscript alphabets show significant difference between experimental groups (P≤0.05)

Table 3: Mean±SD of miotic index score in testes of rats at 3 and 30 days after clenbuterol administration.

Days after clenbuterol administration					
		3rd days		30th days	
Groups	Control	Clen low*	*Clen high	Clen low	Clen high
Miotic index	3.32±0.12[a]	2.04±0.20[b]	1.04±0.20[c]	2.30±0.13[b]	1.50±0.24[d]

*Clen Low: Clenbuterol was gavaged with the dose of 2.5 mg/kg/day
*Clen high: Clenbuterol was gavaged with the dose of 8 mg/kg/day
[a,b,c,d]different superscript alphabets show significant difference between experimental groups (P≤0.05)

The mean height of the epithelium in the different groups are shown in the **Table 4**. This parameter was significantly decreased in all treatment groups on days 3 and 30 in comparison with the control group (P<0.05). At day 3, the height of seminiferous tubules epithelium at Clen-high dose was significantly lower than the Clen-low dose group (P≤0.05). At day 30, in the Clen-high dose group, the epithelium height was significantly higher than the same group at day 3th day. This point indicates a partial improvement in tissue texture in this group. Conversely, in the Clen-low dose group seminiferous tubules epithelium height at day 30 showed a significant reduction in compared to day 3.

Treatment with clenbuterol reduced significantly seminiferous tubules diameter at day 3 and 30 after administration (**Table 5**). Decrease in seminiferous tubules diameter in the Clen-high dose group on day 3 was greater than the Clen low dose group, whereas this parameter increased in the Clen-high dose group at day 30. Seminiferous tubules diameter in the Clen-low dose group at day 30 was not significantly different from day 3.

Blanco et al. (2002) showed that long-term administration of clenbuterol in male pigs causes degenerative change in testicular tissue and formation of giant cells in the seminiferous tubules epithelium that indicates impaired spermatogenesis [16]. Also, there are other studies that describe pathological lesions induced by clenbuterol in the testes [17, 26]. At the present study, degenerative lesions including disorganization of germ cells, reduction of their number and hollow spaces within the lining of seminiferous tubules were observed following use of clenbuterol.

Table 4: Mean±SD of the seminiferous epithelial height (µm) in testes of rats at 3 and 30 days after clenbuterol administration.

Days after clenbuterol administration					
		3rd days		30th days	
Seminiferous epithelial height	Control	Clen low*	*Clen high	Clen low	Clen high
	59.37±4.23[a]	49.67±4.46[b]	25.81±6.57[c]	39.00±3.52[d]	46.87±4.05[b]

*Clen Low: Clenbuterol was gavaged with the dose of 2.5 mg/kg/day
*Clen high: Clenbuterol was gavaged with the dose of 8 mg/kg/day
a,b,c,ddifferent superscript alphabets show significant difference between experimental groups (P≤0.05)

Table 5: Mean±SD seminiferous tubules diameter (µm) in testes of rats at 3 and 30 days after clenbuterol administration.

Days after clenbuterol administration					
		3rd days		30th days	
Seminiferous tubules diameter	Control	Clen low*	*Clen high	Clen low	Clen high
	266.66±24.61[a]	187.22±9.29[b]	168.66±11.59[c]	186.95±5.26[b]	190.90±9.41[d]

*Clen Low: Clenbuterol was gavaged with the dose of 2.5 mg/kg/day
*Clen high: Clenbuterol was gavaged with the dose of 8 mg/kg/day
[a,b,c,d]different superscript alphabets show significant difference between experimental groups (P≤0.05)

The effect of clenbuterol on testicular tissue seems to be due to binding to beta-adrenergic receptors, which has been shown to abound on Leydig, Sertoli and germ cells [14,27]. Even beta2-adrenoceptors have been identified on sperm cells [15]. Binding of clenbuterol to these receptors result in alterations within the cell signaling pathways that eventually disrupt the normal function of the cells, which is seen in electron microscopy images as pathological changes such as the creation of intracellular vacuoles [17, 26].

In the present study, doses of 2.5 and 8 mg/kg body weight were used to evaluate the effects of clenbuterol on testicular tissue. Dose of 2.5 mg/kg is NOEL dose of clenbuterol based on researches in mice and means the highest dose of drug without side effects. Dose of 8 mg/kg is equal one tenth of LD50 of clenbuterol in male mice which was considered as higher than usual in our study [18]. In farm animals, clenbuterol is usually studied in a different way as feed additive and consumed dose generally was different from the amount of clenbuterol used in the present study. For example, Biolati et al., (1994) had added 1ppm of clenbuterol to the ration to evaluate the pathological lesions in pigs. In sports and informal sources, the dose of clenbuterol in an adult man has been stated between 20 and 40 micrograms daily. However, some studies in farm animals had chosen a dose of 20 micrograms per kg body weight, which is still different from the baseline dose of clenbuterol in our study [28]. Li and Zhu (2015) used doses of 0.4, 2 and 18.5 mg/kg for investigation of clenbuterol effects on the testis, and their justification was that these doses were equal to 0.002, 0.01 and 0.1of the clenbuterol LD50 in rats respectively [26]. In the study of Lynch et al. (1996), a dose of 2 mg/kg was used and no reason for choosing this dose of clenbuterol was mentioned [29].

Interestingly, in the present study, deleterious effects of clenbuterol on testicular morphometric parameters and histopathologic changes in low dose was higher than high dose in the long term, whereas in the short term, deleterious effects of high dose was more severe than low dose especially in the spermatogenesis percentage index. Frazer et al. (1986) showed that repeated use of clenbuterol reduces the number of beta-2-adrenoceptors in brain cells [30]. In another study, the usual dose of clenbuterol (20 μg/kg) in female calves for 40 days significantly reduced the number of beta-2 adrenoceptors in the uterus and ovary [31]. It has been shown that the cellular response to repeated administration of natural or synthetic ligands decreases very rapidly as a result of the decrease in the number of receptors, and this is usually a dose-dependent process, and as the dose increases, this process occurs more rapidly [32, 33]. In Ma and Zhou's (2010) study, short-term administration of clenbuterol significantly increased StAR gene expression, whereas in long-term administration there was no effect on StAR levels [34]. In the present study, it seems that administration of high dose of clenbuterol induced its deleterious effects by binding to its receptors in the short term and following the reduction of beta-2 adrenoceptors in this group over time, its deleterious effects decreased. Whereas, in the low dose group, the receptors were less affected by down-regulation mechanism and increased the detrimental effects of clenbuterol over time. More examinations are required for confirmation of this theory.

4. Conclusion

According to the results, clenbuterol as a long act beta-2-adrenergic drug could impact fertility by induction of degenerative changes in testicular tissue. This impact is under the influence of dose and duration of consumption so that, the NOEL dose need to long duration for disclose clenbuterol side effects in testes. While, high doses of clenbuterol induce their adverse effects in short time after administration and over time due to the down-regulation of its receptors on testicular tissue the detrimental effects of it are reduced.

Conflict of interest

The authors declare to have no conflict of interest.

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