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Effect of clenbuterol hydrochloride on weight gain and histological lesions in mice

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Abstract

Clenbuterol hydrochloride (CLB) is a growth-promoting β-agonist in animals for supply, but its illicit use has generated repercussions on public health. A biological model with mice was developed to evaluate the effect of CLB on weight gain and histological lesions. Mice were fed rabbit meat, which was previously supplemented with CLB. Body weight was recorded 35 days post-exposure; muscular and serum concentration of CLB was obtained through the ELISA test, and tissues were collected from liver and heart for histopathological analysis. Values obtained from the experimental animals (G1 and G2) were analyzed by a completely randomized experimental design with two treatments (n = 10), subjected to an analysis of variance and comparison of means with the Tukey test ($p < 0.05$). There was an increase of 7 g in body weight in G1, compared to 4.0 g in G2. Liver weight was 2.58 g and 1.79, respectively ($p < 0.05$). In G1, CLB concentration in muscle was 5324 $pg.g^{-1}$, and 4378 $pg.g^{-1}$ in blood serum. Only histological changes were observed in the tissues of G1 mice. Liver showed cellular swelling, moderate mitosis, pyknosis and hydropic degeneration; in addition, fiber thickening, pleomorphism and nuclear atypia were observed in the heart. CLB contributed to weight gain in exposed mice and caused structural alterations in liver and heart.

Keywords: clenbuterol hydrochloride, toxicity, mice, ELISA.

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Efecto del clorhidrato de clembuterol en la ganancia de peso y lesiones histológicas en ratones

Resumen

El clorhidrato de clembuterol (CCL) es un β-agonista promotor del crecimiento en animales para abasto, pero su uso ilícito ha generado repercusiones en salud pública. Se realizó un modelo biológico con ratones, con el objeto de evaluar el efecto del CCL sobre la ganancia de peso y las lesiones histológicas que ocasiona. Los ratones fueron alimentados con carne de conejo, que previamente fue suplementada con CCL. Treinta y cinco días posexposición se registró el peso corporal; se obtuvo la concentración muscular y sérica de CCL a través de la prueba de ELISA, y se colectaron tejidos (hígado y corazón) para análisis histopatológico. Los valores obtenidos de los animales experimentales (G1 y G2) se analizaron mediante un diseño experimental completamente al azar con dos tratamientos (n = 10), sometidos a un análisis de varianza y comparación de medias con la prueba de Tukey ($p < 0,05$). Se registró un incremento de peso corporal de 7 g en el G1, contra 4,0 g del G2. El peso del hígado fue de 2,58 g y 1,79, respectivamente ($p < 0,05$). En el G1 la concentración muscular de CCL fue 5324 pg g^{-1} y en suero sanguíneo de 4378 pg g^{-1}. Solo se observaron cambios histológicos en tejidos de los ratones del G1. El hígado

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mostró tumefacción celular, mitosis moderada, picnosis y degeneración hidrópica; en corazón, engrosamiento de fibras, pleomorfismo e hileración nuclear. El CCL favoreció el incremento de peso en los ratones expuestos, y provocó alteraciones estructurales en hígado y corazón.

Palabras clave: clorhidrato de clenbuterol, ELISA, toxicidad, ratones (Fuente: MeSH).

Efeito do cloridrato de clembuterol no aumento de peso e lesões histológicas em ratos

Resumo

O cloridrato de clembuterol (CCL) é um β-agonista promotor do crescimento em animais para abate, porém o seu uso ilícito tem gerado repercussões em saúde pública. Fez-se um modelo biológico com ratos, com o objeto de avaliar o efeito do CCL sobre o ganho de peso e as lesões histológicas que ocasiona. Os ratos foram alimentados com carne de coelho, que previamente foi suplementada com CCL. Trinta e cinco dias pós-exposição se registrou o peso corporal; obteve-se a concentração muscular e sérica de CCL através da prova de ELISA, e foram coletados tecidos (fígado e coração) para análise histopatológica. Os valores obtidos dos animais experimentais (G1 e G2) foram analisados mediante um desenho experimental completamente ao acaso com dois tratamentos (n = 10), submetidos a uma análise de variações e comparação de médias com a prova de Tukey ($p < 0,05$). Registrou-se um aumento de peso corporal de 7 g no G1, contra 4,0 g do G2. O peso do fígado foi de 2,58 g e 1,79, respectivamente ($p < 0,05$). No G1 a concentração muscular de CCL foi 5324 pg g^{-1} e em soro sanguíneo de 4378 pg g^{-1}. Somente foram observadas mudanças histológicas em tecidos dos ratos do G1. O fígado apresentou inchaço celular, mitose moderada, picnose e degeneração hidrópica; no coração, engrossamento de fibras, pleomorfismo e filamento nuclear. O CCL favoreceu o aumento de peso nos ratos expostos, e provocou alterações estruturais em fígado e coração.

Palavras chave: cloridrato de clembuterol, toxicidade, ratos, ELISA.

Introduction

Clenbuterol hydrochloride (CLB) [4-Amino-alpha-[(tert-butylamino)methyl]-3,5-dichlorobenzyl alcohol hydrochloride] is a synthetic β-adrenergic agonist, white or slightly yellow, with a molecular weight of 313.65 (1-3). It is used as a bronchodilator in human and veterinary medicine. Its use as an anabolic agent mainly in cattle is currently illegal. It has been shown to increase protein synthesis by up to 15%, and to decrease body fat deposition by 18% (4,5).

CLB causes hypertrophy and muscle neoformation by means of glycogenolysis—nitrogen accumulation for amino acid conformation—and glycolysis—fat degradation—, which increases the size of muscle cells, mainly in skeletal muscle (6-8). Its growth-promoting effect is mediated by direct stimulation of β_2 adrenergic receptors located in the muscle tissue and variation in plasma concentrations of catabolic or anabolic hormones, such as glucocorticoids, growth hormone or insulin (9,10).

The effect of CLB on the endocrine system occurs by hormone release through catecholamines, which release insulin, increase glucagon and stimulate the release of adrenocorticotropic hormone, somatotropin, and gonadotropin. This decreases fat deposition by increasing metabolic activity and energy expenditure with increased thermogenesis; part of the ingested energy prevents fat formation by increasing the level of cyclic adenosine monophosphate (AMP) in the adipose tissue. Adenosine triphosphate (ATP) is converted into cyclic AMP that activates protein kinases, which, by phosphorylation, stimulate an intracellular lipase that transforms triglycerides into fatty acids and glycerol (9-11).

Although the use of CLB has been banned in several countries, including Mexico (3,12), its clandestine administration in the feeding of cattle has been a public health problem worldwide (3,5,11,13,14), due to the consumption of meat and viscera (liver) contaminated with this substance. This causes pathological processes in humans, manifested by muscular tremor, excessive sweating, limb numbness, headache, and tachycardia (8,15).

The study and analysis by Jiménez et al. (16) reported 2130 cases of CLB intoxication in Mexico between 2002 and 2008. Jalisco, the Federal District, Guanajuato, Zacatecas and Michoacán registered the most cases. Jalisco was the state with the highest number of cases (35.25%), followed by the Federal District (23.94%), and cases per year had increased significantly. However, the study did not report the levels detected in affected patients neither did it establish the dose patients were exposed to and which caused abnormality. Likewise, possible histological lesions in tissues where CLB can accumulate have not been evaluated. Thus, the objective of this study was to evaluate the effect of CLB on weight gain and histopathological alterations in the liver and heart of mice exposed to rabbit meat contaminated with CLB as the sole source of food.

Materials and methods

Prior to this study, a biological model was designed, in which rabbits were given CLB (in "mineral salt" form) diluted in drinking water at a dose of 16 μg/kg of CLB (1.1815 μg/L/day), for a period of 35 days, at the end of which they were sacrificed. All muscle tissue was collected and its CLB concentration was determined to be 7619 pg g^{-1}.

Experimental animals

For this research, 20 6-weeks old male BALB/c4 mice were used; they were placed in individual cages complying with comfort and animal welfare conditions. The research complied with the ethical, technical, scientific, and administrative guidelines of the Ministry of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA) (17) for animal research.

Experimental procedure

Mice were randomly distributed into two groups (G1 and G2); both groups received rabbit meat (dehydrated at 80°C) as their sole source of food, at a rate of 3.5 g per day, and distilled water on free demand, as follows:

G1: rabbit meat containing 7619 pg g^{-1} of CLB (n = 10).
G2: control group, rabbit meat with 0 residues of CLB (n=10).

Sample collection and processes performed

Thirty-five days after initiating the experiment, the weight of the mice was measured. They were sacrificed according to the guidelines established by SAGARPA (18), and the whole blood was collected in tubes without anticoagulant. An anatomical-pathological study was performed to evaluate macroscopic changes; liver and heart samples were collected and fixed in 10% buffered formalin (10:1). Hematoxylin-eosin staining was used for histological evaluation.

The ELISA test

CLB concentrations were obtained using the ELISA test (RIDASCREEN® Clenbuterol Fast, R-Biopharm AG, Darmstadt, Germany), using 20 µl of stratum from the muscle tissue sample or blood serum. Absorbances were read with a 450 nm filter in a BioTek plate reader, and the values obtained were expressed as pg g^{-1} (11,19).

Data analysis

For result analysis, a one-way analysis of variance was performed using a completely randomized design, in which treatment was considered as the main source of variance, with two treatments of 10 experimental units in each. Tukey's multiple comparison test ($p<0.05$) was applied to the variables that showed significant differences (20).

Changes observed in the anatomical-pathological and histological studies of tissues and organs collected from the two groups were evaluated qualitatively and by assessment based on the degree of alteration in each tissue, as described by Ke et al. (21).

Results and discussion

In the applied biological model, after the inclusion of meat with a CLB concentration of 7619 pg g^{-1} for G1 and 0 residues of CLB for G2 as the sole source of food, G1 animals showed a greater increase in body weight than G2 mice ($p < 0.05$) (Table 1).

Strydom et al. (22) evaluated growth performance and carcass characteristics in cattle after supplying different β-agonists (zilpaterol, ractopamine, and CLB) and found better feed conversion and weight gain with lower fat deposition in the carcasses of CLB-treated animals compared to the control group ($p < 0.05$), followed by animals treated with zilpaterol. However, it has been shown that the eating of CLB-contaminated meat produces adverse effects on human health, due to cardiovascular system stimulation. Without recording the exact concentration, it has been reported that the consumption of meat contaminated with a "moderate" dose of CLB produces numbness in hands, muscular tremors, nervousness, and headaches, while at "higher" doses, it results in tachycardia, which can lead to myocardial necrosis (14,23,24).

In the immunoenzymatic assay, mean serum concentrations of CLB were 0.00 pg g^{-1} and 4378 ± 437.57 pg g^{-1} for G 2 and G 1, respectively ($p<0.05$). Blood serum and urine should be used to detect CLB in animals for supply prior to slaughter, which would allow for a quarantine period necessary for possible elimination (7,11). Due to the prolonged use of CLB in different species for supply (cattle, pigs and poultry), the presence of this substance can be detected in the retina, hair, and feathers, due to its affinity for melanin found in these structures, which has an indicative value for its illegal use (8,25).

Due to the presence of CLB in the muscle tissue of rabbits that were fed to mice and the subsequent detection of CLB in the muscle of these mice (5324 pg g^{-1}), it was possible to confirm the capacity of CLB for biological retention and deposition in white tissues. It has been established that the residence time and kinetics of CLB in blood serum can be determined over a period of four to six days, which will continue with detectable concentrations depending on the dose and administration time. This represents a risk factor of intoxication for consumers, as observed in the food chain of this experiment with the administration of CLB-contaminated meat. At this point, it is important to consider the particularity of the chemical structure of CLB (chlorine content), given its residence time in consumable tissues, which is 56 to 60 days in the liver and six days in the muscle tissue (25).

Furthermore, the results obtained support that CLB is a stable particle at dehydration temperature (80°C), with a prolonged action. According to the *Codex Alimentarius* (26), which establishes a concentration of 2000 pg g^{-1} as the "maximum residue limit" for CLB in blood serum, the detected values of CLB in G1 mice are considered high. This confirms the accumulation capacity of this

Table 1. Weight of mice (BALB/c4) after administration of CLB-contaminated meat

Group	Initial weight (g)	Final weight (g)	Body weight gain (g)
G1 (7619 pg g^{-1} of CLB)	$18.0^{a} \pm 0.52$	$25.0^{a} \pm 0.52$	7.0^{a}
G2 (Control: 0 residues)	$18.1^{a} \pm 0.51$	$22.1^{b} \pm 0.50$	4.0^{b}

Different letters per column indicate values that statistically differ (Tukey, $p < 0.05$).

substance in the food chain and its residual effect, given that the experimental animals were the second link in the food chain.

At necropsy, no evident macroscopic lesions were observed in the animals of the two treatment groups, except for increased muscle mass with lower fat deposition in G1 mice. The mean liver weight was 1.79 g and 2.58 g for G2 and G1 mice, respectively ($p < 0.05$).

The histopathological study of heart muscle in G1 mice showed muscle fiber thickening (10 of 10), pleomorphism and nuclear swelling (Figure 1). In the liver of CLB-treated animals, the observed lesions were tumefaction and moderate-to-diffuse hydropic degeneration, mitosis, pyknosis, and megalocytosis with hepatocyte megakaryosis (Figure 2).

The changes observed in heart muscle are consistent with that reported by Van Vleet and Valentine (27), who consider fiber swelling and thickening as a process of muscular hypertrophy, which was consistently present only in CLB-treated animals. The alterations observed in heart suggest that CLB acts on specific β_2-type receptors localized in this tissue, inducing structural changes, and its effect is greater on skeletal muscles (23,28). Based on its chemical structure, the action of CLB has been related to catecholamine action, capable of interacting with β_2-type adrenergic receptors, activating the GS protein system associated with adenylyl cyclase, which plays an important role in the entry of calcium into the cell and intervenes in cellular protein synthesis (3,6,29).

Figure 1. Histological cut of heart muscle from G1 mice, evidence of muscular hypertrophy denoted by pleomorphism and nuclear swelling, as well as fiber thickening (arrows). Staining: Hematoxylin and eosin. 10X

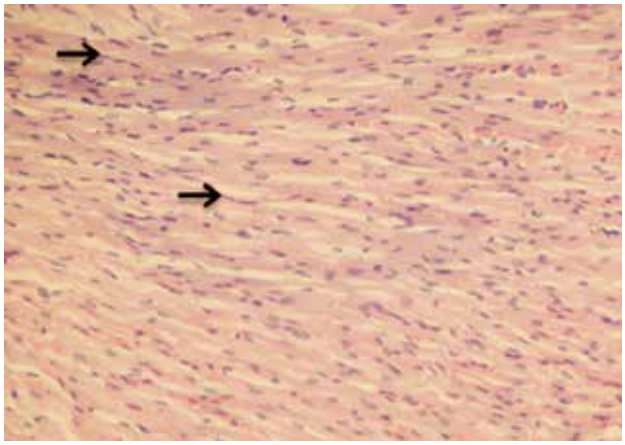
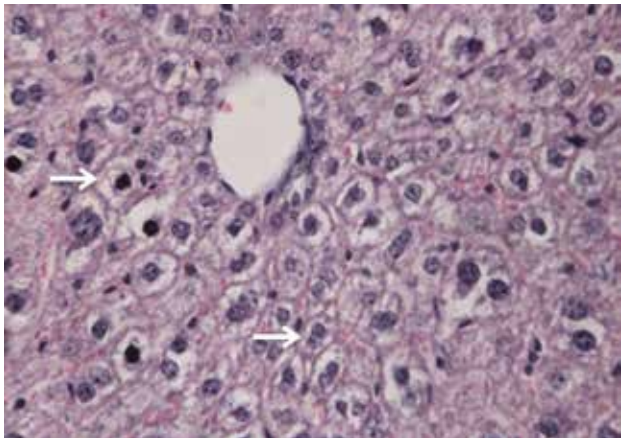


Figure 2. Histological cut of liver from G1 mice, evidence of swelling and moderate hydropic degeneration, mitosis and hepatocyte pyknosis (arrows). Staining: Hematoxylin and eosin. 20X



In the liver of mice exposed to CLB (10 of 10), significant alterations were observed, evidencing the toxic effect of CLB (10,28). CLB has a prolonged action in the organism; it is stored mainly in liver and kidney and is metabolized into hydroxyclenbuterol and glucuronic conjugates by N-oxidation reactions (13,28). Zalco, Bories and Tulliez (29) report that CLB metabolites, such as N-hydroxylamine and N-nitroso clenbuterol, have toxic properties that are hazardous for both animal and human health.

Badino, Odore and Re (9), after administering 30 and 50 µg of CLB/kg/day to rabbits, observed fetotoxic effects (delayed ossification and cleft palate). Another study reports that the possible toxic effect is mainly due to an effect of adrenergic stimulation, rather than to a genotoxic effect (30).

No apparent symptoms were observed in animals under treatment (except for increased activity or some nervousness). However, the intoxication symptoms presented by two patients after eating liver and meat contaminated with CLB, reported by Carrola et al. (31), were muscle tremor, nausea, and incoordination. At auscultation, patients showed increased heart rate (90/min), increased blood pressure (140/80 mmHg), while hematological examination evidenced leukocytosis (12.1-12.2 $\times$ 10^9 L^{-1}), accompanied by neutrophilia (9.3-10.1 $\times$ 10^9 L^{-1}), hyperkalemia (2.7-2.8 mmol L^{-1}), and hyperglycemia (9.5-12.1 mmol L^{-1}).

In bovines, and perhaps in other species for supply, the clandestine and irrational use of CLB continues to occur in an alarming way (3,11,29,31). Therefore, regulations must be updated and applied for the benefit of public and animal health. Similarly, permanent programs must be implemented to monitor, control and eradicate the use of this substance, while proposing alternatives (32) for good animal production practices.

Conclusions

Using a CLB concentration three times higher than the maximum limit established as toxic, a greater weight gain was observed in mice that were exposed to CLB-contaminated meat. CLB favors weight gain in mice; however, the histopathological alterations observed in liver show a hepatotoxic effect.

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