Evaluation of clenbuterol-induced changes in blood biochemical parameters in white mice

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ABSTRACT
Clenbuterol is a β₂-adrenergic agonist which is illegally used in animal production as a promoting agent. The accumulation of clenbuterol in edible tissues has potentially adverse effects that can induce to long-term metabolism disorders after clenbuterol withdrawal. Purpose of this study is to analysis effects of clenbuterol residues on some blood parameters in white mice. 68 mice were divided into 3 groups: control group (n = 27), clenbuterol group A (CA) (n = 20) and clenbuterol group B (CB) (n = 21). Clenbuterol groups were received clenbuterol 2 µg/kg orally once daily. After 8 weeks, mice in group A discontinued receiving clenbuterol and mice in group B continued receiving clenbuterol until 16 weeks. Liver, kidney and blood samples were collected at week 8 and week 16 for biochemistry parameter tests: plasma glucose, triglyceride, total cholesterol, total protein, creatinine concentrations; plasma AST and ALT activities. Results showed that oral clenbuterol administration at dosage 2 µg/kg once daily for 8 weeks significantly decreased ALT activities and elevated glucose concentrations. Clenbuterol-induced dyslipidemia might reverse after withdrawing using clenbuterol while hyperglycemia might potentially make a progress.

1. INTRODUCTION
Clenbuterol is a bronchodilator which is FDA-approved for treatment chronic obstructive pulmonary disease (COPD) in horse and non-lactating cattle¹,². Clenbuterol binds selectively to β₂-receptor in smooth muscles and consequently causing muscle relaxation. The recommended dose of clenbuterol for treatment COPD in veterinary is 0.8µg/kg twice daily¹,³. At doses 5-10 folds higher than therapeutic dose, clenbuterol induces weight gain, increases muscle and decreases adipose tissue mass⁴. Due to its anabolic effects, clenbuterol is illegally used in animal production as a promoting agent and is abused by sportsmen for bodybuilding¹,⁵,⁶.

The chemistry structure of clenbuterol is similar to derivations from endogenous catecholamines. The 3,5-dichloro groups prevent clenbuterol from being metabolized by catechol-O-methyltransferase (COMT), which results in its long half-life and its accumulation in animal tissues⁷. Researches on clenbuterol pharmacokinetics in men and animals reported that clenbuterol was well-absorbed when administered orally and excreted predominantly via urine¹,⁵. After administering orally for 21 consecutive days at dosage of 10-20 times higher than the therapeutic dose, it was observed that clenbuterol accumulated in liver, bile, eye and other edible tissues⁷. Additionally, clenbuterol residues in animal tissues were found to be unaffected by boiling water and a range of cooking processes such as boiling, frying, roasting⁵. Consumption of clenbuterol-contaminated food can lead to

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poison for human with many toxicity symptoms such as dizziness, tachycardia, tachypnea, tremors, vertigo, etc. These symptoms occurred between 0.5-3 hours after meal and maintained for 3-5 days in patients.

To control better the misuse of clenbuterol, the maximum residue limits (MRLs) of clenbuterol for liver and kidney in cattles and horses are accepted at 0.5µg/kg. However, clenbuterol is used illicity as a repartitioning agent and clenbuterol-contaminated food induced several acute intoxications in human. In these cases, it was reported that clenbuterol residues in edible tissues were higher 2,000 times than MRLs, and the amount of clenbuterol consumed was estimated about 230-300µg.

Previous studies evaluated the prolonged effects of clenbuterol on animals (mice, rats, pigs) at high concentrations (20-2,000 µg/kg body weight), and the results showed that clenbuterol affected protein, glucose and lipid metabolism. However, these studies were conducted for a short time. On the other hand, researches on effects of low-dose clenbuterol on animals were limited and only studied about repartitioning effects. The aim of this study was to analyze the effects of low-dose clenbuterol on biochemistry parameters in white mice.

2. MATERIALS AND METHODS

2.1 Reagents and Samples

Clenbuterol hydrochloride was purchased from Sigma-Aldrich. 68 male Swiss Albino mice and animal food was purchased from Pasteur Institute (Ho Chi Minh city, Viet Nam). Reagent kits for determination of plasma AST, ALT activities and plasma glucose, total protein, total cholesterol, triglyceride, HDL-cholesterol and LDL-cholesterol were the gifts from ELI-TechGroup. Reagent kits for plasma creatinine tests were purchased from AMS.

2.2 Animal care

68 male Swiss Albino mice (18-23 g) were bred in-house in a conventional colony, housed in controlled temperature (25°C-27°C) with relative humidity from 50-60% and a 12h-dark/light cycle. Water and animal food were given to the mice ad libitum. All the procedures were carried out under Animal Research Advisory Committee Guidelines. This study was performed with permission from scientific committee of university of medicine and pharmacy at Ho-chi-minh city.

2.3 Study design

Mice were pre-fed for 2 days before the experiment to allow adaptation. 68 mice were randomly divided into 3 groups: control group (n = 27), clenbuterol group A (CA) (n = 20) and clenbuterol group B (CB) (n = 21). The average body weights between 3 groups were not significantly different at the beginning of experiment. All mice were weighted daily during the experimental period.

Clenbuterol hydrochloride was dissolved in distilled water to obtain clenbuterol concentration at 200 ng/ml and then was administered orally to 2 study groups CA and CB at dosage of 2 µg/kg body weight once daily. Control group were received equivalent volume of water instead of clenbuterol solution in the same manner.

After 8 weeks, CA group discontinued receiving clenbuterol solution while CB group continued receiving 200 ng/ml clenbuterol solution at dosage of 2 µg/kg body weight once daily until 16 weeks.

2.4 Sampling

Liver, kidney and whole blood samples were collected at the end of week 8 and week 16. Mice were anaesthetised by CO₂. Whole blood samples were obtained from the heart, then added into EDTA blood tubes and centrifuged at 4,500 x g for 10 min at room temperature. Plasma samples were used immediately for biochemistry parameter tests or stored at -5°C until analysis (within 7 days).

2.5 Analysis method

Plasma creatinine concentrations were determined by the rate-blanked Jaffe assay (AMS). AST and ALT activities were determined by the IFCC method without pyridoxal phos-
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2.6 Statistical Analyses

All statistics were performed using R software (R Development Core Team, New Zealand, version 3.3.2). Results are presented as mean ± SEM. Student’s t-test was used to evaluate the difference between groups. P-values < 0.05 were considered significant for all tests.

3. RESULTS

3.1 Effects of clenbuterol on body mass and the weights of viscera organs

It was observed that clenbuterol induced a rapid and significant increase in body mass after administering for 1 week (p < 0.01). Body weight gains between study groups and control group were significantly different within 8 weeks, but no significant difference was found in the next 8 weeks (from week 9 to week 16). From week 11 to week 16, the weekly body weight gains in the CB group unchanged, while the weekly body mass gains of CA group continued increased progressively (see Figure 1).

The kidney and liver weights at the end of week 8 and week 16 are shown in Table 1. Mean liver weight in study groups was greater than the control group at week 8 (CA+CB: 1.39 ± 0.06 g, control: 1.29 ± 0.04 g, p = 0.17), which slightly increased in the liver mass-to-body weight ratio (CA+CB: 4.42 ± 0.17%, control: 4.45 ± 0.07%, p = 0.86). However, these differences were not significant. At week 16, liver mass decreased in both CA and CB groups, but only the significant difference between CB group and control group was observed (p = 0.004). Mean kidney mass was significantly affected by clenbuterol after 8 weeks of experiment, but no significant difference in kidney weights among 3 groups was found at week 16 (see Table 1).

![Figure 1. Weekly body weight gains of mice during experiment (16 weeks).](image-url)
3.2 Effects of clenbuterol on biochemistry parameters

There was a significant increase in glucose concentration after using clenbuterol for 16 weeks between CB group and control group. Furthermore, the value of mean glucose concentration of CB group was over the normal threshold (106-278 mg/dl), suggested that clenbuterol caused hyperglycemia in mice. After a cessation of clenbuterol for 8 weeks, mean glucose concentration in CA group was found over the normal threshold and significantly higher than control group. However, there was no significant difference in mean total protein concentrations between study groups and control group at week 8 (CA+CB: 58.27 ± 4.06 mg/dl, control: 55.94 ± 2.15 mg/dl, p = 0.66), while significant increase in total protein concentration was found after using clenbuterol for 16 weeks in CB group versus control group (59.31 ±1.42 mg/dl, control: 51.50 ± 2.02 mg/dl, p = 0.66), while significant increase in total protein concentration was found after using clenbuterol for 16 weeks in CB group versus control group (59.31 ±1.42 mg/dl, control: 51.50 ± 2.02 mg/dl, p < 0.05). No abnormal value of plasma triglyceride, total cholesterol, HDL-cholesterol and LDL-cholesterol concentrations were found in control mice at the end of week 8 and week 16. Compared to control group at week 16, plasma triglyceride and total cholesterol concentrations from group CB-mice could not be affected by clenbuterol, while mean plasma triglyceride concentration from group CA-mice was significantly higher. Mean HDL-cholesterol concentration decreased about 36% and mean LDL-cholesterol concentration increased about 34% after using clenbuterol for 8 weeks, but these changes were not significant comparing with control group. After withdrawing clenbuterol for 8 weeks (from week 9 to week 16), HDL-cholesterol and LDL-cholesterol values of mice in CA group were similar to that in control group. On the contrary, at week 16, in CB group, there was a significant decline in HDL-cholesterol value (CB: 53.55 ± 10.16 mg/dl, control: 90.97 ± 12.64 mg/dl, p = 0.033) and an increase of 18% in LDL-cholesterol value (CB: 73.95 ± 7.62 mg/dl, control: 62.51 ± 7.68 mg/dl), suggested that clenbuterol caused dyslipidemia in mice.

The results showed that clenbuterol had no effects on plasma AST activities (CA+CB: 2.77 ± 0.42 µkat/L, control: 2.76 ± 0.28 µkat/L, p = 0.6471) but significantly decreased ALT activities after administering for 8 weeks (CA+CB: 1.20 ± 0.12 µkat/L, control: 1.69 ± 0.15 µkat/L, p = 0.0171). Repeated oral administration of clenbuterol for a duration of 16 weeks induced significant decreases in values of both plasma AST activities (CB: 1.68 ± 0.12 µkat/L, control: 2.42 ± 0.06 µkat/L, p = 0.0028) and plasma ALT activities (CB: 1.08 ± 0.06 µkat/L, control: 1.69 ± 0.14 µkat/L, p = 0.0016). Additionally, plasma ALT activities decreased after a cessation of clenbuterol for 8 weeks (CA: 1.06 ± 0.08 µkat/L, control: 1.69 ± 0.14 µkat/L, p = 0.0012).

The values of plasma creatinine concentrations of clenbuterol-treated mice determined at the end of week 8 showed an increase by 9.5% versus the control mice (CA+CB: 0.81 ± 0.03 mg/dl, control: 0.74 ± 0.02 mg/dl, p = 0.0684). Noticeably, the values of control mice were in normal range (0.5-0.8 mg/dl) while that of clenbuterol-treated mice were beyond the normal threshold. However, this difference was not significant. No significant difference was found among 3 groups at week 16 (Table 2).

Mean plasma creatinine concentrations were

<table>
<thead>
<tr>
<th>Organs</th>
<th>Week 8</th>
<th>Week 16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>Group CA + CB</td>
</tr>
<tr>
<td>Liver</td>
<td>(n = 13)</td>
<td>(n = 13)</td>
</tr>
<tr>
<td></td>
<td>1.29 ± 0.04</td>
<td>1.39 ± 0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.27 ± 0.01</td>
<td>0.32 ± 0.02*</td>
</tr>
</tbody>
</table>

*: p-value < 0.05

Table 1. Liver and kidney weights of mice at the end of week 8 and week 16
still higher than the normal threshold when administering clenbuterol repeatedly until 16 weeks, but would return to normal range after 8-week withdrawal of clenbuterol.

Table 2. Values of biochemistry parameters of mice at the end of week 8 and week 16

<table>
<thead>
<tr>
<th>Biochemistry parameters</th>
<th>Week 8</th>
<th>Week 16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group (n = 13)</td>
<td>CA + CB group (n = 13)</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>196.01 ± 24.46</td>
<td>333.23 ± 33.4</td>
</tr>
<tr>
<td>Total protein (mg/dL)</td>
<td>55.94 ± 2.15</td>
<td>58.27 ± 4.06</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>154.48 ± 9.31</td>
<td>166.54 ± 10.56</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>90.12 ± 10.61</td>
<td>100.20 ± 8.72</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>112.17 ± 17.99</td>
<td>71.77 ± 13.84</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>64.81 ± 22.11</td>
<td>86.66 ± 10.61</td>
</tr>
<tr>
<td>AST activity (µkat/L)</td>
<td>2.76 ± 0.28</td>
<td>2.77 ± 0.42</td>
</tr>
<tr>
<td>ALT activity (µkat/L)</td>
<td>1.69 ± 0.15</td>
<td>1.20 ± 0.12</td>
</tr>
<tr>
<td>Creatinin (mg/dL)</td>
<td>0.74 ± 0.02</td>
<td>0.81 ± 0.03</td>
</tr>
</tbody>
</table>

*: p-value < 0.05, clenbuterol-treated groups vs. control group.

4. DISCUSSION

The effects of β₂-agonists on glycemia were controversial. According to previous studies, after binding to β₂-receptor, β₂-agonists activated several sub-units of G protein family which consequently causes glucose-stimulated and hormone-stimulated insulin secretion\textsuperscript{13,22}. When using at dosage of 10mg/kg and 100mg/kg consecutively in 30 days, clenbuterol induced a significant decline in glucose concentrations in mice\textsuperscript{23}. According to Shujia J. Pan et al., clenbuterol increased insulin action and the rate of glycogen synthesis in skeletal muscles and adipose tissues, which improved insulin resistance in concious obese Zucker rats\textsuperscript{17}. However, in many case reports, glucose concentration increase was reported in patients who were hospitalized after clenbuterol administration or consumption of clenbuterol-contaminated food\textsuperscript{1,2,24}. In our study, it was found that clenbuterol administration at dosage 2µg/kg for 8 weeks induced an augment of 70% in plasma glucose concentration in mice. In addition, there was a potential risk of clenbuterol-induced hyperglycemia in spite of the 8-week cessation. These results suggested that prolonged administration of clenbuterol would induce hyperglycemia in mice and this effect might be irreversible.

This could be explained since clenbuterol induced noradrenaline and glucocorticoid...
(cortisol and corticosterone) release which induced hyperglycemia\(^{25-27}\), clenbuterol could indirectly cause an increase in blood glucose concentration. A research of Wei Guo et al. showed that clenbuterol depleted glycogen deposition in hepatocytes and affected genes that are involved with glucose metabolism\(^{16}\). Therefore clenbuterol could induce a progress of hyperglycemia after 8-week withdrawal in mice.

The effects of clenbuterol and its mechanism on lipid metabolism were unclear. Hye-Kyeong Kim et al. reported that clenbuterol increased the rate of lipolysis and concomitantly decreased the rate of lipogenesis in adipose tissues\(^{28}\). In addition, it was found that clenbuterol down-regulated lipoprotein lipase, which hydrolyzes triglyceride in lipoproteins into glycerol and fatty acid\(^{29}\). In the present study, we observed that when orally administering clenbuterol to mice for 16 weeks at dosage 2 \(\mu\)g/kg, mean LDL-cholesterol concentrations were slightly increased and mean HDL-cholesterol concentrations were significantly decreased when comparing with control group, without changes in triglyceride and total cholesterol concentrations.

In mice, HDL-cholesterol amounted to approximately 70% and LDL-cholesterol amounted to about 25% of total total serum lipoprotein\(^{30}\) therefore the HDL-cholesterol/LDL-cholesterol ratio in mice is > 1. In our study, the HDL-cholesterol/LDL-cholesterol ratios of control mice at the end of week 8 and week 16 were clearly > 1, while those of clenbuterol-treated mice were < 1, suggested that repeated administration of clenbuterol for a long time would lead to dyslipidemia. Additionally, the HDL-cholesterol/LDL-cholesterol ratio in clenbuterol-treated mice at the end of week 16 (0.72) was lower than that at the end of week 8 (0.83). However, after withdrawing clenbuterol for 8 weeks, HDL-cholesterol and LDL-cholesterol concentrations of mice in CA group were similar to control group and the HDL-cholesterol/LDL-cholesterol ratio was > 1, suggested that dyslipidemia in mice was improved.

It has been well known that clenbuterol increases skeletal muscle and decreases adipose tissue mass when using at high dose\(^{4}\). The anabolic effect of clenbuterol was reported even when using at therapeutic dose\(^{19,20}\). In our study, clenbuterol was found to cause a significant increase in total plasma protein concentration after using for 16 weeks. This result was comparable to previous researches on mechanism of clenbuterol on protein metabolism, which clearly demonstrated that clenbuterol increased the rate of protein synthesis and decreased the rate of protein degeneration\(^{15,31,32}\).

Researches on effects of clenbuterol on liver and hepatic function were limited. According to a study conducted by T. Gojmerac et al., clenbuterol caused a significant increase in ALT activities and a slight decrease in AST activities in female pig after repeated administration for 28 days at dosage 10 \(\mu\)g/kg twice daily\(^{11}\). In patients who were hospitalized due to adverse effects of clenbuterol, increase in AST and ALT activities were reported\(^{1,33}\). Wei Guo et al. showed that clenbuterol decreased apoptosis in hepatocytes\(^{16}\). On the contrary, our study showed that oral administration of clenbuterol at dosage 2 \(\mu\)g/kg in mice decreased ALT activities and had no effect on liver weights after using for 8 weeks. We considered that the effects of clenbuterol on plasma ALT activities were irreversible as after the 8-week cessation. In addition, at week 16, plasma AST activities and liver weights significantly decreased in clenbuterol-treated mice when comparing with control mice. This result was comparable to a previous study, which showed a decrease in both AST activities and liver weight when using clenbuterol at high dose (100 mg/kg) in mice for 30 days\(^{23}\).

According to Wilfred A. Nix et al., increase in glycemia might lead to vitamin B\(_6\) deficiency, which serves as a coenzyme of AST and ALT\(^{34}\). In both clenbuterol-treated groups, at the end of week 16, hyperglycemia was reported. Hence, we consider that AST and ALT activity decrease might be relative with clenbuterol-induced hyperglycemia. Further experiments need to be conducted to clarify the effects of clenbuterol on liver function as well as its mechanism.
For the effects of clenbuterol on renal function, results from previous researches were limited and unclear. Increase in plasma creatinine concentrations, even end-stage renal disease, was reported in patients who were hospitalized due to adverse effects of clenbuterol\textsuperscript{35,36}. However, creatinine concentrations of some patients were in normal range\textsuperscript{1,35}. Clenbuterol was found to accumulate in kidney and its residues were found on day 28 after clenbuterol withdrawal\textsuperscript{4,7}. Consequently, it was considered that prolonged administration of clenbuterol had potential risk on renal function.

In our study, we found that plasma creatinine concentrations from group CB-mice were higher than normal threshold after clenbuterol withdrawal at week 16, while plasma creatinine concentrations from group CA-mice were in normal range. It was suggested that clenbuterol residues might reversibly affect renal function. However, since increase in plasma creatinine concentrations might be due to increase in protein degeneration, it is not sure whether clenbuterol induce renal dysfunction or not. Further analysis need to be carried out to confirm the effects of clenbuterol on kidney and to clarify its mechanism.

In conclusion, oral clenbuterol administration at 2µg/kg daily in mice (equivalent to 0.17µg/kg daily in human)\textsuperscript{37} for a long time might affect kidney and liver function as well as glucose and lipid metabolism. Clenbuterol-induced dyslipidemia might reverse after withdrawing using clenbuterol while clenbuterol-induced hyperglycemia might potentially make a progress. Thus, prolonged consumption of clenbuterol-contaminated food or misuse clenbuterol for body-building have potential risks for dys-metabolism and organ dysfunctions. Since both hyperglycemia and dyslipidemia are risk factors of cardiovascular diseases, it is necessary to pay more attention to the use of clenbuterol in animal product and body-building areas.

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REFERENCES


