Osteopathy in Broiler Chicks Fed Toxic Mimosine in *Leucaena leucocephala*

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Further studies of mimosine toxicity in broiler chicks were done to clarify a possibility of osteopathy.

The mineral content and density of femur and the strength, ductility, and toughness for the index of mechanical properties significantly decreased in the 1% mimosine group, compared with those in the control and restricted groups. The stiffness had a decreasing tendency in the 1% mimosine group. Consequently, it was concluded that chicks fed *ad libitum* a 1% mimosine diet for 12 days developed osteopathy.

The bone mineral density and the strength of the restricted group were lower than those of the control group, and those of the 1% mimosine group were still lower than those of the control group. Contents of pyridinoline and deoxypyridinoline in the excrement were significantly higher in the restricted group than those in the control group, but the contents in the 1% mimosine group were significantly lowest among the groups. Osteopathy in chicks fed mimosine, therefore, seemed to be done by loss of appetite and changing to a low turnover of bone caused by mimosine.

Key words: mimosine; osteopathy; broiler chicks; bone mineral content

*Leucaena leucocephala* of tropical and subtropical legume family contains mimosine, β-{N-(3-hydroxy-4-oxo-pyridyl)}-α-aminopropionic acid of a toxic substance. It has been reported that feeding of large quantities of this plant bring about alopecia, growth inhibition, and enlargement of thyroid gland in livestock.1-14 The mimosine toxicity therefore has been impeding the use of *L. leucocephala* for fodder. To overcome those problems, it becomes necessary to study of the mechanism that evokes mimosine toxicity. We have observed a decrease in food intake and body weight gain, specific leg weakness symptoms, sitting down and cramping, and enlargement of kidneys in male broiler chicks (7 days old) fed a 1% mimosine diet.5

Judging from specific leg weakness symptoms appeared in chicks fed mimosine, there is a possibility of osteoporosis. Osteoporosis is a group of bone diseases caused by deficiency of estrogen,6-9 low turnover by aging,9 hyperparathyroidism,10 hyperthyroidism,11 and chronic kidney failure.12 It has also been reported that rats received subcutaneous injection of corticosterone develop osteoporosis either with increase of osteoclast or with decrease of osteoblast, and with concomitant decrease of food intake and body weight gain.13-17 Since the various symptoms observed in the chicks fed 1% mimosine diet closely resemble those in the rat, there is a chance that chicks fed a 1% mimosine diet may develop osteoporosis. It has been reported that deficiency of Vitamin D3 likewise caused hypocalcemia and osteomalacia.18,19 As enlargement of kidneys is observed in the chicks fed 1% mimosine diet, it is considered that decrease of 1,25-(OH)2-Vitamin D3 due to kidney failure might be responsible for the development of osteomalacia. The purpose of this study thus is to give further insight of the mechanism of mimosine toxicity. For this purpose, the mineral content and mechanical properties of the femur of broiler chicks fed 1% mimosine diet were measured.

Materials and Methods

Preparation of crude mimosine. Crude mimosine was prepared from crushed seeds as described previously.5 Approximately 3500 g of crushed seeds usually yielded 50 g of crude mimosine of 87.5% purity.

Experimental animals. Hatched Cobb strain male broiler chicks were kept on commercial broiler feed (metabolisable energy 3100 kcal/kg, crude protein 23.5%) for 1 week before commencing the experiment. Male broiler chicks (7 days old) were divided into three test groups, the 1% mimosine group fed *ad libitum* a commercial diet containing 1% crude mimosine, a restricted group fed a normal diet in the same amount with one fed the 1% mimosine group, and a control group fed *ad libitum* commercial diet, and maintained on the various diets for 12 days. One group contained 5 chicks. Commercial diet consisted of maize (62.24%), bean cake (26.40%), fish meals (7.00%), animal oils and fats (1.90%), sodium chloride (0.25%), calcium carbonate (0.88%), calcium phosphate (0.98%), methionine (0.15%), and premix (vitamins and minerals) (0.20%). Contents of calcium, magnesium, zinc, and iron in the commercial diet were 7.93, 1.73, 0.11, and 0.23 mg/g, respectively.

Chicks in all experiments were housed individually in controlled room temperature (25 ± 2°C) and lighting cycle for 12 h, and were allowed free access to water.

Body weight and food intake of the chicks were measured every day, and excretions were collected on the last
day. Serum was collected by centrifugation of the blood at 12000 rpm for 20 min. Liver, kidney and adrenal gland were excised and their total weights were determined. Right and left femurs were removed, and stored at \(-60^\circ C\).

Ryukyu University’s guide for the care and use of laboratory animals was followed in this study, so chicks were killed under ether anesthesia.

**Measurement of bone mineral content in the femur.** Dual energy X-ray absorptiometry (DXA) was used for the measurement of mineral content and density of the left femur. Left femur was scanned by DCS-600 (Aloka Company) at 1 mm pitch. Scanned graphic images were processed for the mineral content and density of the left femur.\(^{21,22}\)

**Analysis of mechanical properties of the femur.** Mechanical properties of the left femur used in the foregoing experiment were analyzed with an experimental compressor (TM-3, Toyo Boldwin Company) and automatic balance record meter. The three-point bending form of the method of Peng et al.\(^{23,24}\) was used in this study. The left femur was turned down part of the femur front, and both ends of the femur was lightly fixed by a thread on the stand. Width of the stand was 14 mm. Conditions for measurement was 40 kgf weight, 10 mm/min sweep speed, and 100 mm/min chart speed. Strength, ductility, and stiffness of the left femur used in the foregoing experiment were measured based on the recorded stress/strain curve.\(^{25,26}\) Toughness of the left femur was measured by integrating the covered area of stress/strain curve by use of image analysis software (NIH image).

**Measurement of corticosterone (CTC) concentration in the serum.** The improved Scott and Dixon's method\(^{27}\) was used for measurement of CTC concentration in the serum. To 1.6 ml of the serum, 0.4 ml of 0.25 N sodium hydroxide and 20.0 ml of dichloromethane were added. The solution was mixed gently, and shaken for 1 min in a stoppered glass tube. After centrifugation (2000 rpm, 10 min), the dichloromethane layer was evaporated under reduced pressure at room temperature, and redissolved in 200 μl of mobile phase (methanol:water=45:55 v/v). A sample of this preparation was put onto a Shim-pack CLC-ODS column (6×150 mm). The HPLC was used a Shimadzu LC-6A with a UV detection at 248 nm wavelength. Separation was done at 30°C with 1.0 ml/min flow rate and a linear elution gradient of methanol from 45 to 83.5%.

**Measurement of Vitamin D\(_3\) and 25-(OH)-Vitamin D\(_3\) concentration in the serum.** To 2.0 ml of serum, 2.0 ml of methanol-isopropanol (90:10 v/v) and 6.0 ml of n-hexane were added, and mixed vigorously for 15 s and 60 s, respectively. The mixture was centrifuged at 1500 rpm for 3 min. The hexane layer was carefully transferred to a glass tube, and evaporated to dryness by flushing N\(_2\) stream. The residue was redissolved in 200 μl of methanol, and analyzed by HPLC analysis.\(^{28}\) A sample of this preparation was put onto a shim-pack CLC-ODS column (6×150 mm). The HPLC was used a Shimadzu LC-6A with a UV detection at 265 nm wavelength. Separation was done at 30°C with 1.5 ml/min flow rate and a linear elution gradient from mobile phase A (methanol:water=85:15 v/v) to mobile phase B (methanol:isopropanol:water=87.5:10:2.5 v/v).

**Measurement of mineral concentrations in the serum and excretion.** The improved forms of Uchida’s method\(^{29}\) and S. L. Gaffin and H. Hornung's method\(^{30}\) were used for measurement of mineral concentrations in the serum and excretion. To 0.5 ml serum or 0.5 g freeze dried excretion in the dried (110°C, 40 min) Unisel (Unisel Decomposition Vessels Ltd),\(^{31}\) 10.0 ml of concentrated nitric acid was added. The vessels were heated at 150°C for 90 min. On addition of 10.0 ml of concentrated hydrochloric acid and 30% hydrogen peroxide (1:1) the mixture solution was evaporated to dryness on a sand bath. To the residue, 10 ml of 3 N hydrochloric acid was added, and the mixture was dissolved by heating. The sample was diluted with distilled water, and the contents of calcium (Ca), magnesium (Mg), zinc (Zn), and iron (Fe) were measured by atomic-absorption frame spectrophotometry (Shimidzu AA-660). The reagents were used all ultra fine grade (Nacalai Tesque Co., Ltd.).

**Measurement of bone metabolism markers.** Contents of pyridoline (Pyd) and deoxypyridinoline (Dpd) as an indicator of bone resorption were measured by use of EIA Kits (Pyrilinks and Pyrilinks-D, Metra Biosystems, Inc., USA). To 0.5 g of freeze dried excretion, 3.0 ml of distilled water was added, and mixed vigorously for 60 s. The mixture was centrifuged at 12000 rpm for 20 min. The supernatant solution was used as the sample solution.

**Statistical Analysis.** Data were tested by one-way analysis of variance followed by inspection of differences between means by Duncan’s new multiple range test throughout these experiments. The different superscript letters in the figures shows statistically significant differences at p<0.01.

**Results**

**Growth and tissues weight**

Body weight and food intake in the 1% mimosine group significantly decreased (p<0.01) from the fourth day and the eighth day, respectively, compared with those in the control group. The restricted group had comparable body weight gain (7.3 g/day) to the 1% mimosine group (Table I). Specific leg weakness symptoms, sitting down and cramping, were shown from the fourth day in the 1% mimosine group. Kidneys weight per 100 g of body weight significantly increased (p<0.01) in the 1% mimosine group by contrast with ones in the control and restricted groups. Enlargement of kidneys was observed only in the 1% mimosine group, but not in the restricted group (Table II).
**Table I.** Food Intake, Body Weight, and Body Weight Gain

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>Restricted diet</th>
<th>1% mimosine diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake</td>
<td>Initial</td>
<td>Final</td>
<td>Final</td>
</tr>
<tr>
<td>(g/day)</td>
<td>15.4±1.6a</td>
<td>19.3±2.5b</td>
<td>16.0±1.4a</td>
</tr>
<tr>
<td>Body weight</td>
<td>Initial</td>
<td>Final</td>
<td>Final</td>
</tr>
<tr>
<td>(g)</td>
<td>91.1±6.8a</td>
<td>92.0±10.0b</td>
<td>93.6±8.0a</td>
</tr>
<tr>
<td>Body weight gain</td>
<td>14.5±4.2b</td>
<td>7.4±0.7b</td>
<td>7.3±3.4b</td>
</tr>
</tbody>
</table>

Means±SD, n=5.

1 Initial day of the experiment, 2 Final day of the experiment
The different superscript letters show statistically significant difference at p<0.01.

**Table II.** Tissue Weight per 100 g of Body Weight

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>Restricted diet</th>
<th>1% mimosine diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g)</td>
<td>4.9±1.1a</td>
<td>4.0±0.3b</td>
<td>4.5±1.0a</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>1.4±0.2b</td>
<td>1.3±0.2</td>
<td>2.3±0.7b</td>
</tr>
<tr>
<td>Adrenal (mg)</td>
<td>24.7±6.7a</td>
<td>23.3±2.1b</td>
<td>31.5±13.9b</td>
</tr>
</tbody>
</table>

Means±SD, n=5.

* Kidney and adrenal mean total weight of right and left ones.
The different superscript letters show statistically significant difference at p<0.01.

**Mineral content and density of the left femur**

Length was significantly shortest in the 1% mimosine group than in the other groups. Width had a tendency to be short in the 1% mimosine group, compared with one in the other groups. The bone mineral content in the 1% mimosine group significantly decreased (p<0.01) by contrast with those in the other groups. The bone mineral density of the restricted group significantly decreased (p<0.01) by comparing with those of the control group, moreover the 1% mimosine group had still significantly (p<0.01) lower density than the restricted group (Table III). Coefficient of variation in this measurement was 1.79%.

**Mechanical properties of the left femur**

Strength of the restricted group significantly decreased (p<0.01) by contrast with the control group, besides the strength of the 1% mimosine group was significantly lower (p<0.01) than one of the restricted group. Ductility and toughness were significantly lowest (p<0.01) in the 1% mimosine group than those in the other groups. The ratios of strength, ductility, and toughness of the 1% mimosine group to those of control group were 41.8, 61.4, and 28.1%, respectively. Stiffness had an decreasing tendency in the 1% mimosine group, compared with the other groups (Table IV).

**Corticosterone (CTC), Vitamin D₃, and 25-(OH)-Vitamin D₃ concentration in the serum**

CTC concentration in the serum significantly increased (p<0.01) in the 1% mimosine and restricted groups, compared with the control group. Vitamin D₃ concentration of serum tended toward an increase in the 1% mimosine and restricted groups by contrast with the control group. 25-(OH)-Vitamin D₃ concentration in the serum significantly increased (p<0.01) in the restricted group, and had an increasing tendency in the 1% mimosine group, compared with the control group (Table V).

**Minerals (Ca, Mg, Zn, and Fe) concentration in the serum and excretion**

In the serum, Ca concentration significantly increased (p<0.01) in the 1% mimosine group, compared with one in the other groups. The Mg concentration was significantly lowest (p<0.01) in the restricted group than the other groups. Zn and Fe concentrations had an increasing tendency in the 1% mimosine and restricted groups by contrast with those in the control group. On the other hand, in the excretion per 1 g, Ca concentration had an increasing tendency in the 1% mimosine and restricted groups, compared with the control group, and Zn and Fe concentrations had an decreasing tendency in the 1% mimosine and restricted groups by contrast with those in the control group (Table VI).

**Contents of bone metabolism markers**

The quantities of Pyd and Pdp in the excrement had significantly (p<0.01) highest in the restricted group than those in the other groups, but significantly (p<0.01) lowest in the 1% mimosine group than those in the other groups (Table VII).
Osteopathy in Broiler Chicks Fed Mimosine

Table V. Corticosteron, Vitamin D₃, and 25-(OH)-Vitamin D₃ in Serum (ng/ml)

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>Restricted diet</th>
<th>1% mimosine diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosteron</td>
<td>18.10±9.83⁵</td>
<td>132.08±15.57⁵</td>
<td>94.90±12.38⁵</td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>282.00±88.16⁹</td>
<td>348.00±90.71⁹</td>
<td>316.90±34.46⁹</td>
</tr>
<tr>
<td>25-(OH)-Vitamin D₃</td>
<td>3.16±1.29⁵</td>
<td>5.21±1.96⁵</td>
<td>3.37±0.99⁹</td>
</tr>
</tbody>
</table>

Means±SD, n=5. The different superscript letters show statistically significant difference at p<0.01.

Table VI. Content of Calcium, Magnesium, Zinc, and Iron in Serum and Excrement

<table>
<thead>
<tr>
<th></th>
<th>Serum (mg/dl)</th>
<th>Excrement (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control diet</td>
<td>Restricted diet</td>
</tr>
<tr>
<td>Calcium</td>
<td>7.26±0.92⁴</td>
<td>7.20±0.49⁴</td>
</tr>
<tr>
<td>Magnesium</td>
<td>2.58±0.59⁴</td>
<td>2.12±0.22⁴</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.33±0.13</td>
<td>0.41±0.18</td>
</tr>
<tr>
<td>Iron</td>
<td>4.08±1.96</td>
<td>7.11±4.59</td>
</tr>
<tr>
<td>Calcium</td>
<td>3.80±0.23</td>
<td>3.88±0.13</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.18±0.00</td>
<td>0.18±0.00</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.28±0.03</td>
<td>0.26±0.05</td>
</tr>
<tr>
<td>Iron</td>
<td>0.15±0.03</td>
<td>0.14±0.04</td>
</tr>
</tbody>
</table>

Means±SD, n=5. The different superscript letters show statistically significant difference at p<0.01.

Table VII. Pyridinoline (Pyd) and Deoxypyridinoline (Dpd) in Excrement (nm)

<table>
<thead>
<tr>
<th></th>
<th>Pyd</th>
<th>Dpd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Restricted</td>
</tr>
<tr>
<td>Diet</td>
<td>638.96±10.61⁵</td>
<td>192.73±4.81³</td>
</tr>
<tr>
<td>1% mimosine</td>
<td>749.77±18.28⁸</td>
<td>209.73±1.40³</td>
</tr>
<tr>
<td>1% mimosine</td>
<td>547.04±30.88⁵</td>
<td>166.97±1.90³</td>
</tr>
</tbody>
</table>

Means±SD, n=5. The different superscript letters show statistically significant difference at p<0.01.

Discussion

Since it seems that leg weakness symptoms that appeared in the mimosine group is caused by a decrease in food intake, this experiment contained the restricted group to find another factor aside from loss of appetite by the action of mimosine. The symptoms were shown in the 1% mimosine group, but not in the restricted group. The 1% mimosine group had loss of body weight gain as well as the restricted group, compared with the control group. It was suggested that leg weakness symptoms was not only caused by loss of appetite but also by other factors. Enlargement of kidneys was only shown in the 1% mimosine group, so mimosine might produce some kidney trouble.

The mineral content and density of femurs and the strength, ductility, and toughness for the index of mechanical properties significantly decreased in the 1% mimosine group, and the stiffness had a decreasing tendency in the 1% mimosine group, compared with the other groups. Osteoporosis has been defined to follow all decreases of the bone mineral content, change of the delicate structure of bone, and increased risk of fracture. This work, however, didn’t find one of them, change of the delicate structure of bone. Therefore it was concluded that the condition of chicks fed ad libi-
tum a 1% mimosine diet for 12 days was suitable for osteopathy. The bone mineral density and the strength of the restricted group were lower than those of the control group, and those of the 1% mimosine group were still lower than those of the restricted group. Osteopathy in chicks fed mimosine, therefore, seemed to be not only caused by loss of appetite, but also an other factor.

Since the value of the 25-(OH)-Vitamin D₃ in the 1% mimosine group was almost equal to that in the restricted group, osteopathy in chicks fed mimosine may be caused by inhibition of activation of Vitamin D₃ and other factors.

The CTC concentration in the serum significantly increased in the 1% mimosine and restricted groups. It seems that osteopathy in chicks fed mimosine is caused by increase of CTC concentration in the serum as well by other factors.

In the report of A. Al Dehneh et al., the CTC concentration in the serum of the goat received intravenous injection of mimosine (200 mg · kg⁻₀.₇₅ · day⁻¹) for 2 days is 8.7 ng/ml in the control group and 6.2 ng/ml in the mimosine group. The mimosine group had an decreasing tendency, compared with the control group. R. Puchala et al. however, the CTC concentration in the serum of the goat received intravenous injection of mimosine (40 mg · kg⁻₀.₇₅ · day⁻¹) for 3 days is 14.0 ng/ml in the control group and 62.0 ng/ml in the mimosine group, and CTC significantly increased in the mimosine group, compared with the control group. Our result agreed with the results of R. Puchala et al. However, the amount of ingested mimosine per metabolic body size is 576.9 mg · kg⁻₀.₇₅ · day⁻¹, so it seemed to be difficult to make the comparison.

Mg, Zn and Fe concentration in the serum had an increasing tendency in the 1% mimosine, compared with control group. M. Smuts et al. has reported that Ca and Mg concentration in the serum of goats receiving an
intravenous injection of mimosine (12.5 mg·kg⁻⁰·⁷⁵/day) for 1 day had an increasing tendency in the mimosine group, compared with those in the control group. The results of M. Smuts et al.³⁹ agreed with our result. R. Puchala et al.¹³ has reported that Mg and Zn concentration in the serum of the goats significantly decreased in the mimosine group, compared with one in the control group, and mimosine toxicity is caused by deficiency of the minerals (Mg and Zn). Our results disagreed with their results.

Contents of Pdy and Dpd as an indicator of bone resorption in the excrement were significantly higher in the restricted group than those in the control group, but the contents in the 1% mimosine group were significantly lower among the groups. This results suggest that there is low turnover of bone. Osteopathy in chicks fed 1% mimosine diet, therefore, seemed to be caused by loss of appetite and switching from normal to low turnover of bone due to the action of mimosine.

Acknowledgment

On this study, we are deeply grateful to Dr. T. Yoshikawa and Dr. M. Isha (Orthopedics of Faculty of Medicine, University of The Ryukyus) for measurement of bone mineral content, and Dr. T. Akinaga (Agricultural Engineering) for measurement of mechanical properties. Furthermore, we are deeply grateful for the generous gifts of chicks and commercial diet from the Okinawan Animal Development Corporation and Ryukyu Kyodo Feed Mfg. Co., Ltd., respectively. This work partly supported by the Sasakawa Scientific Research Grant from The Japan Science Society.

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