EFFECTS OF PROTEIN VERSUS CALCIUM SUPPLEMENTATION ON BONE METABOLISM AND DEVELOPMENT IN FLUORIDE-EXPOSED OFFSPRING RATS FED PROTEIN- AND CALCIUM-DEFICIENT DIETS

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SUMMARY: The effects of supplemental protein (Pr) versus calcium (Ca) beginning at postnatal day 50, 70, 90, and 110 on bone metabolism and development in fluoride (F)-exposed offspring rats fed Pr- and Ca-deficient diets were studied. Ingestion of F together with the Pr- and Ca-deficient diets significantly inhibited the growth of bone, markedly altered bone structure, and resulted in low Ca and P in bone, which was significantly offset by Pr and Ca supplementation. Moreover, the activity of serum alkaline phosphatase (ALP) and serum tartrate resistant acid phosphatase (StrACP) increased significantly, and the serum calcitonin (CT) level in serum was significantly decreased with high F in the Pr/Ca-deficient group. A Ca-supplemented diet obviously reduced F toxic effects on the activity of serum ALP and StrACP and enhanced the CT level. Additionally, with Pr supplementation, the bone carboxyglutamate protein (BGP) level in serum was efficiently stabilized, which is beneficial for the process of bone turnover. These findings indicate that adequate or supplementary ingestion of dietary factors such as Pr or Ca may significantly reduce toxic effects of F on bone.

Keywords: Bone metabolism; Calcitonin; Calcium supplementation; High fluoride; Nutritional deficiency; Protein supplementation; Rat bone metabolism; Serum alkaline phosphatase.

INTRODUCTION

It is known that a nutritionally rich diet may play an important role in alleviating fluoride (F) intoxication. The association between nutrition factors and fluorosis recovery has been demonstrated by epidemiological investigations comparing high F with nutritionally deficient areas and experimental studies on the effects of protein (Pr) and calcium (Ca) supplementation on F toxicity. Our previous research on fluorosis in goats has provided evidence that nutritional deficiency may aggravate fluorosis, and that Pr supplementation has an ameliorating effect. Others, however, hold that endemic fluorosis is primarily a Ca-deficiency disease that is mainly prevented by supplemental Ca. Therefore the present comparative study was designed to investigate therapeutic effects of supplemental Pr versus Ca on the development and metabolism of bone in offspring rats exposed to F.

MATERIALS AND METHODS

Establishment of animal model: Forty adult Wistar albino rats with similar ability in activity, weighing about 180 g each, were obtained from the Experimental Animal Center of Shanxi Medical University. After one week in quarantine, one male and three females were placed in a cage together for mating. Once the vaginal plug was established, the females were separated and...
moved to separate cages. All the pregnant females were divided randomly into four groups as shown in the protocol in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein (%)</th>
<th>Calcium (%)</th>
<th>Energy (MJ/kg)</th>
<th>NaF (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr/Ca-deficient control</td>
<td>11.99</td>
<td>0.28</td>
<td>12.7986</td>
<td>0</td>
</tr>
<tr>
<td>LPr+LCa+HiF</td>
<td>11.99</td>
<td>0.28</td>
<td>12.7986</td>
<td>221.05</td>
</tr>
<tr>
<td>HiPr+LCa+HiF</td>
<td>27.12</td>
<td>0.29</td>
<td>12.7974</td>
<td>221.05</td>
</tr>
<tr>
<td>LPr+HiCa+HiF</td>
<td>11.95</td>
<td>2.07</td>
<td>12.2480</td>
<td>221.05</td>
</tr>
</tbody>
</table>

In this experiment, all rats were kept in spacious rat housing maintained under standard temperature (22–25°C), 12/12-hr light/dark cycle, ventilation, and hygienic conditions. The study design was approved by the Institutional Animal Care and Use Committee of China.

After weaning, offspring rats received similar treatment as their mothers up to their age of day 50, 70, 90, and 110.

**Biochemical examination:** At postnatal days 50, 70, 90, and 110, six rat pups were randomly selected from each group. After a 24-hr food-and-water fast, they were anesthetized with 20% urethane (ethyl carbamate, NH₂COOC₂H₅) solution. Blood was collected from the eyeball, and centrifuged for 15 min at 3000 rpm. The supernatant was collected as serum and stored at –80°C for further study. The tibia and femur were collected, measured for weight and length, and then stored at –80°C. Additionally, the left tibia was retained and fixed in 10% formalin modified solution for morphology study at postnatal day 110.

The level of serum Ca and P, and the activity of serum tartrate-resistant acid phosphatase (StrACP) and alkaline phosphatase (ALP), and the serum level of calcitonin (CT) and bone carboxyglutamate protein (BGP) were determined with the reagent kit provided by the Nanjing Jianchen Biological Institute. All samples were run at the same time to minimize variation.

**Bone mineral content (BMC) in femur:** The femurs were heated in an oven at 105°C and crushed. One gram of femur was put into a crucible at room temperature and then transferred into a muffle-furnace oven for ashing at 550°C for 4 hr, and finally cooled for 30 min. The calculation of BMC was calculated by: (weight of combustion crucible and bone ash – crucible weight) / dry weight of sample.

**Statistics and data processing:** Data were statistically analyzed by Student’s t test, using SPSS 11.5 software, and expressed as mean±SE with p<0.05 considered statistically significant.

**RESULTS**

**Bone development and morphology:** The body weights of the offspring rats, the lengths of their femurs, and serum protein levels are listed in Tables 2, 3, and 4, respectively. Table 5 shows the results of trabecula bone volume (TBV), cortical bone width (CtWi), bone-trabecular thickness (BTTh), bone-trabecular separation
(BTSp), mineral apposition rate MAR, and double mineralizing surface (DMSu) of the tibias at postnatal day 110.

### Table 2. Effects of Pr and Ca on body weight (g) of rats (n=6; mean±SE)

<table>
<thead>
<tr>
<th>Group</th>
<th>50th day</th>
<th>70th day</th>
<th>90th day</th>
<th>110th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr/Ca-DefC</td>
<td>54.8±1.4</td>
<td>62.0±6.7</td>
<td>97.3±5.0</td>
<td>106.4±3.7</td>
</tr>
<tr>
<td>LPr+LCa+HiF</td>
<td>32.8±1.4</td>
<td>60.0±5.7</td>
<td>61.9±6.3</td>
<td>69.9±5.6</td>
</tr>
<tr>
<td>HiPr+LCa+HiF</td>
<td>102.3±3.0</td>
<td>127.8±3.6</td>
<td>127.9±2.9</td>
<td>166.3±5.8</td>
</tr>
<tr>
<td>LPr+HiCa+HiF</td>
<td>39.8±2.6</td>
<td>57.3±1.7</td>
<td>68.2±4.9</td>
<td>89.8±4.2</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01 compared with the Pr/Ca-DefC group, † p<0.05, †† p<0.01 compared with the HiF group.

### Table 3. Effects of Pr and Ca on length (mm) of femur of rats (n=6; mean±SE)

<table>
<thead>
<tr>
<th>Group</th>
<th>50th day</th>
<th>70th day</th>
<th>90th day</th>
<th>110th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr/Ca-DefC</td>
<td>17.69±0.73</td>
<td>19.28±0.60</td>
<td>24.50±0.34</td>
<td>24.56±0.33</td>
</tr>
<tr>
<td>LPr+LCa+HiF</td>
<td>15.48±0.59</td>
<td>19.62±0.75</td>
<td>19.77±0.50</td>
<td>22.02±0.64</td>
</tr>
<tr>
<td>HiPr+LCa+HiF</td>
<td>20.19±0.75</td>
<td>23.47±0.62</td>
<td>24.26±0.77</td>
<td>25.89±0.70</td>
</tr>
<tr>
<td>LPr+HiCa+HiF</td>
<td>14.83±0.75</td>
<td>18.03±0.50</td>
<td>19.83±0.50</td>
<td>23.16±0.74</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01 compared with the Pr/Ca-DefC group, † p<0.05, †† p<0.01 compared with the HiF group.

### Table 4. Effects of Pr and Ca on serum total Pr (g/L) of rats (n=6; mean±SE)

<table>
<thead>
<tr>
<th>Group</th>
<th>50th day</th>
<th>70th day</th>
<th>90th day</th>
<th>110th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr/Ca-DefC</td>
<td>39.72±4.20</td>
<td>43.74±3.60</td>
<td>40.65±3.20</td>
<td>46.59±2.0</td>
</tr>
<tr>
<td>LPr+LCa+HiF</td>
<td>38.18±2.0</td>
<td>39.28±1.7</td>
<td>40.74±1.4</td>
<td>44.11±2.0</td>
</tr>
<tr>
<td>HiPr+LCa+HiF</td>
<td>44.77±2.1</td>
<td>48.63±2.4</td>
<td>51.03±1.8</td>
<td>48.68±1.5</td>
</tr>
<tr>
<td>LPr+HiCa+HiF</td>
<td>32.47±2.8</td>
<td>42.20±3.2</td>
<td>39.90±2.1</td>
<td>52.71±2.1</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01 compared with the Pr/Ca-DefC group, † p<0.05, †† p<0.01 compared with the HiF group.

### Table 5. Effect of Pr and Ca on tibia histomorphometry of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>TBV (%)</th>
<th>CTWi(µm)</th>
<th>BTTh(µm)</th>
<th>BTSp(µm)</th>
<th>MAR(µm/d)</th>
<th>DMSu(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr/Ca-DefC</td>
<td>4.23±1.4</td>
<td>359.77±55.7</td>
<td>66.88±5.7</td>
<td>193.23±17.2</td>
<td>1.54±0.3</td>
<td>16.48±5.3</td>
</tr>
<tr>
<td>LPr+LCA+HiF</td>
<td>14.43±8.4</td>
<td>290.69±60.4</td>
<td>78.2±9.2</td>
<td>261.58±24.8</td>
<td>1.03±0.2</td>
<td>9.53±3.3</td>
</tr>
<tr>
<td>HiPr+LCA+HiF</td>
<td>9.42±2.9</td>
<td>321.14±57.9</td>
<td>60.8±4.7</td>
<td>160.33±16.3</td>
<td>1.86±0.2</td>
<td>10.61±4.9</td>
</tr>
<tr>
<td>LPr+HiCA+HiF</td>
<td>12.98±3.5</td>
<td>308.22±48.9</td>
<td>66.54±4.8</td>
<td>184.25±13.5</td>
<td>1.18±0.1</td>
<td>13.11±3.4</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01 compared with the Pr/Ca-DefC group, † p<0.05, †† p<0.01 compared with the HiF group.

### Table 6. Effects of Pr and Ca on BMC (%) of rats (n=6; mean±SE)

<table>
<thead>
<tr>
<th>Group</th>
<th>50th day</th>
<th>70th day</th>
<th>90th day</th>
<th>110th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr/Ca-DefC</td>
<td>47.37±0.7</td>
<td>51.22±0.7</td>
<td>51.50±0.3</td>
<td>51.35±0.6</td>
</tr>
<tr>
<td>LPr+LCA+HiF</td>
<td>47.88±1.7</td>
<td>50.43±1.3</td>
<td>48.19±1.5</td>
<td>49.11±0.7</td>
</tr>
<tr>
<td>HiPr+LCA+HiF</td>
<td>53.09±1.0</td>
<td>55.35±0.8</td>
<td>52.90±1.0</td>
<td>51.85±0.4</td>
</tr>
<tr>
<td>LPr+HiCA+HiF</td>
<td>49.72±2.6</td>
<td>51.03±0.6</td>
<td>53.72±0.5</td>
<td>53.91±0.7</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01 compared with the Pr/Ca-DefC group, † p<0.05, †† p<0.01 compared with the HiF group.

**Bone mineral content:** The bone mineral content (BMC) and bone Ca and P in each group of rats are listed in Tables 6–8, respectively.
Biochemical parameters: The activity of ALP and StrACP, and the level of BGP and CT in serum are listed in Tables 9–12.
DISCUSSION AND CONCLUSION

Effects of Pr and Ca on the bone development and morphology in fluorotic rats:
It has been well documented that protein (Pr) and even amino acids can play an important role in reducing the toxic effects of F.3,4,10,11 In the present study, compared with the Pr/Ca-DefC group, growth of rats from LPr+LCa+HiF was significantly inhibited at the postnatal day 50, 90, and 110, while in comparison with the LPr+LCa+HiF group, the body weight of rats in the HiPr+LCa+HiF group was obviously enhanced. These results are very consistent with previous studies.17 However, there was no significant effect in the LPr+HiCa+HiF group. Interestingly, the same trends occurred in the changes of femur length. The possible explanation may be that protein supplementation, to some extent, complemented the over-consumption of the protein during the development period, which was confirmed by the increased serum protein level in the HiPr+LCa+HiF group.

Protein establishes the structural foundation and framework of the bone in preparation for the deposition of minerals.7 Earlier studies showed that excessive F ingestion significantly decreased the protein content, resulting in adverse changes in the histology of both hard and soft tissues.12,13 In the present study, rats fed the Pr/Ca-deficient diet with high F for 110 days exhibited marked alteration in bone structure. Significant increase in the volume, thickness, and separation of trabecular bone and a decrease in mineral apposition rate and double mineralizing surface of tibia were the major changes as compared to the Pr/Ca-deficient control. Treatment with Ca supplementations led to an obvious recovery in thickness and separation of trabecular bone. However, a highly significant reversal of fluorosed tibia was obtained in all parameters except cortical bone width and double mineralizing surface in comparisons with the LPr+LCa+HiF group. This finding supports the above opinion about protein curbing F toxicity.

Effects of Pr and Ca on the bone mineral content in fluorotic rats: Previous studies have demonstrated that F can not only influence the molecular structure of collagen, an important framework protein in bone,7 but can also disorder its gene expression.14-16 Meanwhile, it has also been confirmed that dissolution of bone salts is accelerated by parathyroid hormone action as a result of F exposure.9,17 These findings imply a low bone mineral deposition, in agreement with the data from the present study. Compared with the Pr/Ca-DefC group, bone mineral contents in LPr+LCa+HiF group were markedly decreased at

| Table 12. Effect of Pr and Ca on serum CT (ng/L) of rats (n=6; mean±SE) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | 50th day        | 70th day        | 90th day        | 110th day       |
| Pr/Ca-DefC       | 610.72±28.4     | 1649.59±166.9   | 563.30±43.6     | 589.99±41.5     |
| LPr+LCa+HiF      | 433.32±47.3     | 586.97±52.0     | 536.54±85.4     | 665.08±27.0     |
| HiPr+LCa+HiF     | 378.19±16.3     | 484.83±31.9     | 298.42±30.3     | 469.85±18.9     |
| LPr+HiCa+HiF     | 805.42±19.4     | 826.00±17.3     | 596.90±68.6     | 625.56±40.6     |

*p<0.05, **p<0.01 compared with the Pr/Ca-DefC group, †p<0.05, ††p<0.01 compared with the HiF group.
the postnatal day 90 and 110, especially for bone P, which showed a significant decrease at day 90. It can be seen from the results that both Pr- and Ca-enriched diets enhanced the bone mineral content including Ca and P. However, Pr showed stronger protective effects during the whole experimental period. These results are well verified by the earlier reports on herbivores.¹⁷

**Effects of Pr or Ca on serum metabolic parameters in rats:** In a previous investigation, we demonstrated adverse effects of F on serum ALP and StrACP in rabbits.¹⁷ As a comparative study, the present experiment shows similar effects in rats. Exposure to high F with Pr and Ca deficiency was found to increase the activities of both of these parameters. Significant decreases in serum ALP and StrACP were found in the fluorotic rats fed the Ca supplemented diet compared with the Pr/Ca-DefC diet, while no significant effect occurred with the Pr supplementation, except in the activity of ALP at day 50 and 70.

BGP, also known as γ-Osteocalcin, and CT (calcitonin) are two important indexes reflecting the process of bone turnover. In this study, the serum level of BGP was obviously enhanced in the offspring rats at postnatal day 70, followed by a significant decrease until day 110 in the LPr+LCa+HiF group in comparison with the Pr/Ca-DefC group, indicating that F caused a high activity of bone transformation in the early developmental stage. However, according to the data from the HiPr+LCa+HiF group, Pr supplementation can efficiently offset abnormal bone metabolism induced by F. On the other hand, F exposure significantly reduced the CT level in serum at the postnatal day 50 and 70 compared with the level in the Pr/Ca-DefC group. Its levels, however, were markedly increased by the Ca-enriched diet. Pr showed only a small role in reversing the F-induced serum CT level. Taken together, these changes in serum metabolic parameters provide supporting evidence for mineral loss in bone caused by F.

In conclusion, this investigation demonstrated that ingestion of F along with a diet deficient in Pr and Ca has adverse effects on bone structure and metabolism. On the other hand, diets enriched with Pr and/or Ca are able to offset or reduce F toxicity. Comparatively, Pr is superior to Ca in improving the growth performance of bone.

**ACKNOWLEDGEMENTS**

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Effects of protein vs Ca supplementation on bone metabolism and development in F-exposed offspring rats fed protein- and Ca-deficient diets
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