EFFECTS OF MALNUTRITION AND SUPPLEMENTED NUTRITION ON NONSPECIFIC IMMUNE FUNCTION CHANGES INDUCED BY FLUORIDE IN RABBITS

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SUMMARY: This study was designed to investigate the protective role of protein (Pr) and calcium (Ca) on effects of fluoride (F) induced in nonspecific immunological function in New Zealand rabbits fed a Pr and a Ca nutritionally deficient (malnutrition) diet. Eighty healthy 30-day-old rabbits (1.07±0.25 kg) were divided randomly into four equal groups of twenty (female: male = 1:1). The four groups were maintained on distilled water and fed for 120 days: (1) a malnutrition control (MC) diet (8.58% Pr, 0.49% Ca = MC group); (2) the MC diet plus HiF (442 mg NaF (=200 mg F ion)/kg diet = HiF group); (3) a Ca deficient diet plus HiPr+HiF (18.41% Pr, 0.46% Ca, plus HiF diet = HiPr group); and (4) a Pr deficient diet plus HiCa+HiF (8.35% Pr, 2.23% Ca, plus HiF diet = HiCa group). Growth of the rabbits was markedly inhibited by HiF ingestion. Pr supplementation significantly alleviated the HiF-induced decrease in rate of growth. Compared to the MC group, the serum total Pr (TPr) and serum albumin (ALB) content in the HiF group were significantly decreased on average by 12.4% and 7.7%, respectively. Pr or Ca supplementation markedly increased serum TPr and ALB content compared to the HiF group. Compared to the MC group, tissue acid phosphatase (ACPase), serum lysozyme (Lys), and serum ACPase concentrations were significantly increased with HiF ingestion but were significantly alleviated by Pr and Ca supplementation. These findings indicate that HiF ingestion seriously damages nonspecific immune function in rabbits and that Pr and Ca can play protective roles against F-induced damage to immune functions.

Keywords: Dietary calcium; Dietary protein; High fluoride; Malnutrition; Nonspecific immunity; Rabbit immune function.

INTRODUCTION

Fluoride (F) is ubiquitous in varying amounts in food and water and is an air pollutant from numerous industrial operations. Exposure to F results in F absorption and transportation via the blood to tissues and organs causing structural changes and disturbance in their functions.1 Thus, in addition to skeletal manifestations,2-4 chronic F poisoning is known to cause a variety of pathological changes in non-skeletal tissues. Structural and functional changes in brain,5-12 reproductive tissues,13-18 thyroid gland,19-25 collagen,26-28 liver,29 kidney,30 and erythrocytes,31 have been reported.

Epidemiological investigations have demonstrated that endemic fluorosis is mainly prevalent in undeveloped countries, particularly areas of malnutrition. Many studies report that protein (Pr) and calcium (Ca) malnutrition aggravates fluorosis,32-35 and that protein and calcium supplementation can alleviate it.33-35 Our previous studies indicate that high F and malnutrition aggravated fluorosis, and Pr supplementation had much better ameliorating effects on fluorosis in goats under pasture conditions.2,3,36 However, some authors report that endemic

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fluorosis is also a Ca paradox disease, and that supplemental Ca has significant preventive effects.\textsuperscript{35, 37} The aim of this study was to investigate supplemented Pr and Ca on growth and general health in rabbits under artificially controlled conditions of Pr and Ca malnutrition and high fluoride exposure.

**MATERIALS AND METHODS**

**Animals:** Eighty healthy one-month-old healthy New Zealand rabbits (female:male = 1:1) weighing 1.07±0.25 kg) were obtained from the Rabbit Breeding Farm of Taiyu Country and kept in a spacious animal house at 22–25°C on a 12-hr light/dark cycle. All the animals were provided a similar diet of the same energy content ad libitum along with low-F distilled water. The study design was approved by the Institutional Animal Care and Use Committee of China.

**Treatment:** Details of the treatment of control and experimental groups are presented in Table 1. The rabbits were divided into four equal groups of twenty: group 1 was fed a Pr and Ca deficient, malnutrition control (MC) diet (8.58% protein, 0.49% calcium = MC group); group 2 was given the MC diet plus NaF (8.58% Pr, 0.49% Ca, plus 442 mg NaF/kg diet = HiF group); group 3 was given a Pr-rich, Ca-deficient diet plus HiF (18.41% Pr, 0.46% Ca, plus 442 mg NaF/kg diet = HiPr group); group 4 was given a Ca-rich plus the Pr-deficient diet and HiF (8.35% protein, 2.23% calcium, plus 442 mg NaF/kg = HiCa group). All the experimental rabbits were maintained on distilled water, and each rabbit was caged separately. The diets were prepared according to physical circumstances during the dry grass season (Table 1).

**Biochemical examination:** On the 30\textsuperscript{th}, 60\textsuperscript{th}, 90\textsuperscript{th}, and 120\textsuperscript{th} day of the feeding trial, eight rabbits were selected randomly from each group and deprived of food for 12 hr. After the rabbits were weighed, blood samples were collected by heart puncture. Serum was collected with centrifugation at 3000 rpm for 10 min and stored at –70°C for analysis. Four rabbits in each group were euthanized by air injection in ear vein on the 30\textsuperscript{th}, 60\textsuperscript{th}, and 90\textsuperscript{th} day, and eight on the 120\textsuperscript{th} day. The spleen and thymus were immediately collected, weighed, and homogenized with 1:99 (w/v) and 1:9 (w/v) 0.9% saline solution at 4°C, respectively. Serum TPr and ALB level, ACPase and lysozyme (Lys) activity, and tissue ACPase activity were determined with the reagent kit provided by the Nanjing Jianchen Biological Institute.

**Pathology examination:** On the 120\textsuperscript{th} day, after the 8 rabbits were sacrificed, the spleen was removed for histological examination and fixed in 10%
formaldehyde solution, embedded in paraffin, serially sectioned at 5 micrometers, and stained with haematoxylin eosin.

**Statistical analysis:** Numerical results are expressed as mean±SD. Statistical analyses were performed by Student’s t test. P<0.05 was considered as significant.

**RESULTS**

Data from the various treatments are summarized in Figures 1 and 2 and Tables 2–8. Figure 1 shows the growth curve of the rabbits.

![Figure 1. Increasing curve of weight in rabbits](image)

*\*p<0.05, **p<0.01 (HiF group compared with MC group); †p<0.05, ††p<0.01 (HiPr group compared with HiF group, HiCa group compared with HiF group).

Tables 2–4 list the protein (Pr), calcium (Ca), and phosphorus (P) levels in the feces, and the serum total Pr and albumin, respectively.

**Table 2.** Pr, Ca, P levels (%) of the feces in rabbits (mean±SD; n=4)

<table>
<thead>
<tr>
<th></th>
<th>60th day</th>
<th>120th day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pr</td>
<td>Ca</td>
</tr>
<tr>
<td>MC group</td>
<td>3.95±0.28</td>
<td>0.83±0.18</td>
</tr>
<tr>
<td>HiF group</td>
<td>4.44±0.28*</td>
<td>0.48±0.02**</td>
</tr>
<tr>
<td>HiPr group</td>
<td>8.91±0.69††</td>
<td>0.79±0.09††</td>
</tr>
<tr>
<td>HiCa group</td>
<td>4.21±0.38</td>
<td>1.63±0.21††</td>
</tr>
</tbody>
</table>

*p<0.05, *p<0.01 (HiF group compared with MC group).
†p<0.05, ††p<0.01 (HiPr group compared with HiF group; HiCa group compared with HiF group).

**Table 3.** Serum TPr level (mg/mL) in rabbits (mean±SD; n=8)

<table>
<thead>
<tr>
<th></th>
<th>30th day</th>
<th>60th day</th>
<th>90th day</th>
<th>120th day</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC group</td>
<td>51.43±5.99</td>
<td>51.64±7.10</td>
<td>49.78±7.22</td>
<td>54.01±11.12</td>
<td>51.71±7.84</td>
</tr>
<tr>
<td>HiF group</td>
<td>48.41±7.43</td>
<td>46.42±11.74</td>
<td>44.43±8.09</td>
<td>41.95±10.24*</td>
<td>45.30±9.38**</td>
</tr>
<tr>
<td>HiPr group</td>
<td>57.12±4.91†</td>
<td>61.23±12.58†</td>
<td>54.99±11.25†</td>
<td>59.73±8.14††</td>
<td>58.27±9.52††</td>
</tr>
<tr>
<td>HiCa group</td>
<td>55.45±11.64</td>
<td>56.20±6.29</td>
<td>54.41±9.74†</td>
<td>54.88±9.01†</td>
<td>55.24±8.93††</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 (HiF group compared with MC group).
†p<0.05, ††p<0.01 (HiPr group compared with HiF group; HiCa group compared with HiF group).
Serum lysozyme (Lys) and acid phosphatase (ACPase) and tissue ACPase activities are listed in Tables 5–8, respectively.

**Table 4. Serum ALB level (mg/mL) in rabbits (mean±SD; n=8)**

<table>
<thead>
<tr>
<th></th>
<th>30th day</th>
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<th>90th day</th>
<th>120th day</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC group</td>
<td>30.05±3.61</td>
<td>31.62±2.52</td>
<td>28.73±4.45</td>
<td>31.21±2.31</td>
<td>30.40±3.37</td>
</tr>
<tr>
<td>HiF group</td>
<td>28.89±2.95</td>
<td>29.10±2.85</td>
<td>25.85±4.96</td>
<td>28.34±2.94†</td>
<td>28.05±3.61**</td>
</tr>
<tr>
<td>HiPr group</td>
<td>31.43±3.51</td>
<td>30.36±2.48</td>
<td>30.21±2.20†</td>
<td>31.72±2.82†</td>
<td>30.93±2.74††</td>
</tr>
<tr>
<td>HiCa group</td>
<td>29.68±3.76</td>
<td>31.95±3.47</td>
<td>27.56±3.46</td>
<td>29.07±2.43</td>
<td>29.56±3.54</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 (HiF group compared with MC group).
†p<0.05, ††p<0.01 (HiPr group compared with HiF group; HiCa group compared with HiF group).

**Table 5. Serum Lys activity (U/mL) in rabbits (mean±SD; n=8)**

<table>
<thead>
<tr>
<th></th>
<th>30th day</th>
<th>60th day</th>
<th>90th day</th>
<th>120th day</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC group</td>
<td>534.3±115.8</td>
<td>355.8±61.7</td>
<td>297.7±110.1</td>
<td>501.1±40.9</td>
<td>422.2±130.3</td>
</tr>
<tr>
<td>HiF group</td>
<td>568.0±119.0</td>
<td>541.0±97.8**</td>
<td>500.0±87.0**</td>
<td>473.9±39.4</td>
<td>520.7±93.7**</td>
</tr>
<tr>
<td>HiPr group</td>
<td>477.0±99.3</td>
<td>455.6±97.7</td>
<td>502.3±86.9</td>
<td>409.9±61.7†</td>
<td>461.2±90.2†</td>
</tr>
<tr>
<td>HiCa group</td>
<td>444.4±104.3†</td>
<td>467.7±96.3</td>
<td>558.5±79.9</td>
<td>489.3±45.0</td>
<td>490.0±91.2</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 (HiF group compared with MC group).
†p<0.05, ††p<0.01 (HiPr group compared with HiF group; HiCa group compared with HiF group).

**Table 6. Serum ACPase activity (U/100mL) in rabbits (mean±SD; n=8)**

<table>
<thead>
<tr>
<th></th>
<th>30th day</th>
<th>60th day</th>
<th>90th day</th>
<th>120th day</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC group</td>
<td>39.82±6.99</td>
<td>37.56±8.63</td>
<td>20.09±6.11</td>
<td>12.11±1.98</td>
<td>27.40±13.33</td>
</tr>
<tr>
<td>HiF group</td>
<td>52.31±9.84*</td>
<td>43.12±8.28</td>
<td>25.27±6.96</td>
<td>16.73±5.62*</td>
<td>34.36±16.12</td>
</tr>
<tr>
<td>HiPr group</td>
<td>54.47±9.94</td>
<td>48.15±8.74</td>
<td>23.72±5.00</td>
<td>17.72±3.39</td>
<td>36.01±17.30</td>
</tr>
<tr>
<td>HiCa group</td>
<td>40.67±10.87†</td>
<td>38.78±6.25</td>
<td>22.97±5.04</td>
<td>17.30±5.02</td>
<td>29.93±12.27</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 (HiF group compared with MC group).
†p<0.05, ††p<0.01 (HiPr group compared with HiF group; HiCa group compared with HiF group).

**Table 7. Thymus ACPase activity (U/gprot) in rabbits (mean±SD)**

<table>
<thead>
<tr>
<th></th>
<th>30th d (n=4)</th>
<th>60th d (n=4)</th>
<th>90th d (n=4)</th>
<th>120th d (n=8)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC group</td>
<td>83.95±3.16</td>
<td>110.83±10.56</td>
<td>100.04±13.17</td>
<td>42.06±10.77</td>
<td>75.79±31.06</td>
</tr>
<tr>
<td>HiF group</td>
<td>101.78±12.13*</td>
<td>127.53±10.61</td>
<td>119.88±13.78</td>
<td>51.18±13.44</td>
<td>90.31±35.86</td>
</tr>
<tr>
<td>HiPr group</td>
<td>80.95±13.03</td>
<td>87.35±18.21††</td>
<td>113.67±4.55</td>
<td>41.04±14.37</td>
<td>72.81±31.52</td>
</tr>
<tr>
<td>HiCa group</td>
<td>85.78±9.41</td>
<td>101.78±9.13†</td>
<td>98.48±17.46</td>
<td>46.94±12.62</td>
<td>75.98±27.50</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 (HiF group compared with MC group).
†p<0.05, ††p<0.01 (HiPr group compared with HiF group; HiCa group compared with HiF group).

**Table 8. Spleen ACPase activity (U/gprot) in rabbits (mean±SD)**

<table>
<thead>
<tr>
<th></th>
<th>30th d (n=4)</th>
<th>60th d (n=4)</th>
<th>90th d (n=4)</th>
<th>120th d (n=8)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC group</td>
<td>72.65±3.21</td>
<td>112.68±13.52</td>
<td>93.53±10.95</td>
<td>99.18±11.17</td>
<td>95.44±16.55</td>
</tr>
<tr>
<td>HiF group</td>
<td>96.91±9.66**</td>
<td>143.10±15.57**</td>
<td>133.08±15.61**</td>
<td>113.88±12.85**</td>
<td>120.17±20.66**</td>
</tr>
<tr>
<td>HiPr group</td>
<td>92.82±16.23</td>
<td>128.78±13.73</td>
<td>117.40±12.40</td>
<td>96.45±8.79††</td>
<td>106.38±18.32††</td>
</tr>
<tr>
<td>HiCa group</td>
<td>89.19±15.73</td>
<td>115.58±10.47††</td>
<td>98.01±8.07††</td>
<td>82.28±13.79††</td>
<td>93.47±17.33††</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 (HiF group compared with MC group).
†p<0.05, ††p<0.01 (HiPr group compared with HiF group; HiCa group compared with HiF group).
Morphological changes in spleen tissue of rabbits on the 120th day are shown in Figure 2.

![Figure 2](image)

**Figure 2.** Morphological changes in spleen tissue of rabbits after 120 days stained with haematoxylin and eosin (HE). A, B, C, and D are sections of spleen tissue from the MC, HiF, HiPr, and HiCa groups, respectively (6.6×40).

As seen in Figure 2, in the MC group (A) the boundary between the cortex and medulla was clear, and considerable amounts of lymphocyte in white pulp and red pulp were observed. In the HiF group (B), rare lymphocytes were erratically arranged, and few lymph nodules were present. In the HiPr and HiCa groups (C and D), Pr and Ca supplementation played a protective role to some degree for F-induced pathological changes in the spleen.

**DISCUSSION**

Growth in the rabbits was significantly inhibited by high F, which is consistent with results of previous studies. Pr and Ca supplementation markedly increased the body weight in the F-intoxicated rabbits, and 18.41% Pr supplementation was more effective than 2.23% Ca supplementation over the entire 120-day treatment period. Serum TPr and ALB contents were significantly decreased by 12.4% and 7.7% on average, respectively, in the HiF group. Previous studies in rats, children, rabbits, and Tuj sheep support these findings. Pr supplementation significantly increased serum TPr and ALB contents by 28.6% and 10.3% on average, respectively. Moreover, Ca supplementation also markedly increased serum TPr contents by an average of 21.9%. The reduction in serum TPr and ALB contents in rabbits induced by F might be due to either increased proteolysis or decreased Pr synthesis. It has been reported that F inhibits Pr
synthesis by weakening the beginning of the peptide chain and by preventing the production of peptide chains in ribosomes. In this study, we observed that the P levels in 96-hr feces samples from the 57th to the 60th day were significantly greater by 12.4% in the HiF group. These results suggest that high F not only increased proteolysis or decreased Pr synthesis but also decreased intestinal absorption of Pr and ultimately reduced serum TPr and ALB levels and caused a lower weight gain of the rabbits.

Ca is widely believed to help alleviate F toxicity by forming insoluble CaF$_2$ in the intestines. Interestingly, we observed that the P levels in 96-hr feces samples from the 57th to the 60th day were significantly greater by 12.4% in the HiF group. These results suggest that high F not only increased proteolysis or decreased Pr synthesis but also decreased intestinal absorption of Pr and ultimately reduced serum TPr and ALB levels and caused a lower weight gain of the rabbits.

Nonspecific immunity plays an important defensive role against the encroachment of exotic matter. Chronic F poisoning is known to injure nonspecific immunity function, and ACPase is a marker enzyme for Lys, whose enzyme quantity and quality determine the immunological function of macrophages. In a study of F effects on the silkworm gut, Chen found an increase in ACPase activity. In the present study, serum ACPase activities were significantly increased by 31.4% on the 30th day and 38.2% on the 120th day in the HiF group, compared with the MC group. The thymus ACPase activities were also markedly increased by 21.2% on the 30th day, and the spleen ACPase activities were significantly increased by 25.9% on average, in the HiF group. Lys is synthesized by macrophage and then rapidly released to the blood. In this study, we observed that serum Lys activity was markedly increased by 23.3% on average in HiF group, compared with MC group. These increases in enzyme activities can be seen as a consequence of disordered free radical metabolism induced by F. Excessive formation of free radicals and lipid peroxidation from fluoride damages tissue, cell membrane, inner structure, and the metabolism of biological molecules. In this study, obvious pathological structural changes were observed in the spleen as seen in Figure 2 (B, C, and D). F-induced damage of macrophage and the spleen as an immune organ, finally caused an excessive release of ACPase and Lys. On the other hand, Lys, as an autophagosome, was also provoked by F, resulting in the marked activity increase of ACPase.

Pr supplementation significantly decreased serum Lys activity by 11.4% on average. Likewise, the spleen and thymus ACPase were markedly decreased by...
11.5% on average and by 31.5% on the 60th day, respectively. Ca supplementation also markedly decreased serum ACPase by 22.3% on the 30th day. Similarly, thymus and spleen ACPase were decreased by 20.2% on 60th day and 22.2% on average, respectively. The ameliorative effect of supplemented Pr or Ca may therefore be due to strengthening of physiological function and resistance.

In conclusion, these findings indicate that excessive F markedly inhibited growth and general health in rabbits and increased their nonspecific immune-related ACPase and Lys activities. Pr and Ca supplementation provided a protective role to some extent on growth and nonspecific immunologic functions induced by F. By comparison, protection by Pr supplementation was better than by Ca in these experiments.

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REFERENCES


Malnutrition and supplemented nutrition effects on F-induced immune function changes in rabbits

Zhou, Wang, Wang, Zhang, Yan, Wang