Abstract. – OBJECTIVE: The aim of this study was to investigate the relationship between the changes in intestinal flora and the occurrence of osteoporosis in rats with inflammatory bowel disease and the improvement effect of probiotics.

MATERIALS AND METHODS: A total of 100 Sprague Dawley (SD) model rats with colitis were selected as research objects. All rats were randomly divided into two groups, including: bowel disease group and osteoporosis group, with 50 rats in each group. Stool samples were collected from all rats, and Lactobacillus, Escherichia coli and Bifidobacteria were cultured and counted. The relationship between the occurrence of related osteoporosis and intestinal flora was analyzed as well. Thereafter, the rats in osteoporosis group were randomly divided into two subgroups, namely, control group (n=25) and observation group (n=25). Observation group was treated with probiotics by gastrogavage, while the control group was treated with the same volume of physiological saline. Next, the changes in serum osteoprotegerin (OPG), osteoprotegerin ligand [receptor activator of nuclear factor-kappa B ligand (RANKL)], procollagen type I carboxy-terminal propeptide (PICP), bone mineral density (BMD), bone alkaline phosphatase (BALP), tartrate-resistant acid phosphatase (TRACP), calcium concentration (Ca), and inflammatory cytokine levels were compared between the two groups after intervention.

RESULTS: Osteoporosis group had significantly more Escherichia coli and notably fewer Lactobacillus and Bifidobacteria than bowel disease group (p<0.05). Pearson correlation analysis revealed that the occurrence of osteoporosis in rats with inflammatory bowel disease was negatively correlated with the count of Escherichia coli, whereas was positively related to the counts of Lactobacillus and Bifidobacteria (p<0.05). Moreover, the levels of serum OPG, PICP, TRACP, and Ca in observation group were remarkably higher than those in the control group (p<0.05). However, the levels of serum RANKL, BALP, interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (INF-γ) were markedly lower than those in the control group (p<0.05).

CONCLUSIONS: Osteoporosis in rats with inflammatory bowel disease has a negative association with the count of Escherichia coli, and a positive correlation with the counts of Lactobacillus and Bifidobacteria. In addition, treatment with probiotics can effectively alleviate osteoporosis symptoms in rats with inflammatory bowel disease by influencing the level of corresponding cytokines.

Key Words: Inflammatory bowel disease, Rats, Intestinal flora, Osteoporosis, Probiotics.

Introduction

Osteoporosis is a kind of disease caused by various factors, such as changes in the fibrous structure of bone tissues and the reduction of bone mass in the whole body. Ultimately, it may increase fracture risk and change the normal load function of bone tissues1. The incidence rate of osteoporosis in many clinical diseases of
the digestive tract is relatively high, including inflammatory bowel disease. However, there is no authoritative explanation for the mechanism of osteoporosis\(^2\). Intestinal flora is a relatively crucial microbial ecosystem in the human body. It has been found to modulate the inflammation, immunity, and metabolic state of the body under ill or healthy state\(^3\). Intestinal flora directly participates in the normal physiological metabolism of the host, and facilitates the development and establishment of the immune system of the host’s gastrointestinal tract. Meanwhile, it can also serve as a physiological barrier to prevent the body from being invaded by pathogens\(^4\). Numerous studies have manifested that there is a certain correlation between bone metabolic diseases and intestinal flora imbalance in patients with intestinal diseases\(^5\). Probiotics can effectively interfere with the composition of intestinal flora, modulate the immune system, repress the synthesis and secretion of proinflammatory factors and efficaciously reduce bone absorption, thus playing an important role in the treatment and prevention of osteoporosis\(^6\).\(^7\). In this study, therefore, the relationship between changes in intestinal flora in rats with inflammatory bowel disease and the occurrence of related osteoporosis and the improvement effect of probiotics were explored. All our findings might provide a reference for clinical application.

**Materials and Methods**

**Experimental Rats**

A total of 100 Specific Pathogen Free (SPF)-grade rats (half male and half female) weighing 180-200 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All rats were kept in separate cages in clean laboratories at 22-25°C. This investigation was approved by the Animal Ethics Committee of Zhejiang Chinese Medical University Animal Center.

**Model Preparation**

Experimental enteritis model\(^8\): all rats were fasted for 36 h, and their stools were completely emptied. After intraperitoneal injection of 2% pentobarbital sodium, 2,4,6-trinitrobenzenesulfonic acid (TNBS) enema was performed. Modeling methods: absolute alcohol and 0.3 mL of 5% (w/v) TNBS were prepared at a proportion of 1:1 and used as enema solution. Next, the enema solution was injected at 8 cm from the anus of rats and kept for about 1 min. Preparation of osteoporosis models\(^9\): ovaries on both sides of the abdominal incision of all rats were removed to establish the osteoporosis model in rats. All rats in osteoporosis group was assigned into two subgroups, namely, control group (n=25) and observation group (n=25), using a random number table.

**Intervention Methods**

After modeling, osteoporosis modeling rats received corresponding intragastric treatment. Briefly, the rats in the observation group underwent enema with 12.5 g/kg probiotics. Meanwhile, those in the control group received enema with the same dose of normal saline. One week later, all rats were executed for the following experiments.

**Cultivation, Identification and Counting of Intestinal Flora**

A total of 0.1 g of stools in rat cecum were first extracted. Subsequently, the collected samples were placed in 9 mL of Ringer diluent (containing 0.1 g of cysteine), stirred and diluted to prepare suspensions with four concentrations of 10\(^{-8}\) g/L, 10\(^{-7}\) g/L, 10\(^{-6}\) g/L, and 10\(^{-5}\) g/L. Then, 100 μg of suspensions were smeared on Lactobacillus, Bifidobacteria and Mac Conkey culture media for 24 h of culture at 37°C. Next, Lactobacillus and Bifidobacteria were cultured using the anaerobic culture method. The morphology of bacterial colonies was observed, and colony formation units were finally counted.

**Observational Indexes**

At 1 day after the last administration, 5 mL of blood was collected from the heart, followed by centrifugation at 3000 r/min for 10 min using a centrifuge (ID-Centrifuge 12 S II). Subsequently, the serum was separated and stored at -75°C in a refrigerator for later detection. Determination of serum indexes: the levels of serum interferon-gamma (INF-γ), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), osteoprotegerin (OPG), and osteoprotegerin ligand [receptor activator of nuclear factor-kappaB ligand (RANKL)] were determined via enzyme-linked immunosorbent assay in strict accordance with the instructions of the kit purchased from Beijing Rongzhi Haida Biotech Co., Ltd. (Beijing, China). Meanwhile, enzyme-linked immunosorbent assay was also adopted to determine the levels of serum bone alkaline phosphatase (BALP), tartrate-resistant acid phosphatase (TRACP), calcium concen-
Intestinal flora in osteoporosis rats

Measurement of bone mineral density (BMD) level: after the rats were killed, Kirschner wires in their body were pulled out, and BMD of the samples was measured with a BMD instrument. Then, the BMD of the femur was measured with a dual-energy X-ray scanner. Finally, the BMD data were determined and recorded.

Statistical Analysis
Statistical Product and Service Solutions (SPSS) 20.00 (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Measurement data were expressed as (x±s) and compared via t-test. Intergroup comparisons were conducted using analysis of variance. Count data were expressed as [n (%)] and examined via chi-square test. Pearson method was adopted to analyze the correlation between the changes in intestinal flora in rats with inflammatory bowel disease and osteoporosis. p<0.05 was considered statistically significant.

Results

Culture Counts of Bifidobacteria, Lactobacillus and Escherichia coli in the Two Groups of Rats
Osteoporosis group exhibited a larger count of Escherichia coli and smaller counts of Bifidobacteria and Lactobacillus than bowel disease group, with statistically significant differences (p<0.05) (Table I).

Relationship Between the Occurrence of Relevant Osteoporosis in Rats With Inflammatory Bowel Disease and the Changes in Intestinal Flora
Pearson correlation analysis manifested that the BMD in rats with inflammatory bowel disease was positively correlated with the count of Escherichia coli (r=0.584, p=0.012), whereas was negatively associated with the counts of Lactobacillus and Bifidobacterium, showing statistically significant differences (r=-0.693, p<0.001; r=-0.656, p<0.001) (Table II and Figure 1A-1C). All those results showed that the occurrence of relevant osteoporosis in rats with inflammatory bowel disease was negatively correlated with the count of Escherichia coli (r=0.584, p=0.012), whereas it was positively associated with the counts of Lactobacillus and Bifidobacterium.

Changes in the Levels of Serum OPG and RANKL in the Two Groups of Rats
The level of serum OPG was markedly higher in rats of observation group than those in control group (p<0.05). However, the level of serum RANKL was notably lower in observation group (p<0.05) (Table III).

Changes in the Levels of Serum PICP and BMD in the Two Groups of Rats
The rats in the observation group had a remarkably higher PICP level than control group (p<0.05). However, no statistically significant difference was observed in BMD level between the two groups of rats (p>0.05) (Table IV).

Changes in the Levels of Serum BALP, TRACP and Ca in the Two Groups of Rats
The levels of serum BALP was significantly lower, while the levels of serum TRACP and Ca were prominently higher in rats of observation group than those in control group (p<0.05) (Table V).

<table>
<thead>
<tr>
<th>Item</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>0.584</td>
<td>0.012</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>-0.693</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>-0.656</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Changes in Serum IL-6, TNF-α and IFN-γ Levels in the Two Groups of Rats

Rats in observation group exhibited evidently lower levels of serum IL-6, TNF-α and IFN-γ than control group, displaying statistically significant differences ($p<0.05$) (Table VI).

**Discussion**

Clinically, various diseases of the digestive tract are accompanied by different degrees of intestinal flora disequilibrium symptoms\(^{11}\). Intestinal microecology is a larger ecosystem of the human body. It can delay aging, inhibit bone calcium loss, contribute to bone health and normal cell metabolism, enhance body defense function, and modulate body immunity\(^{12}\). Bowel disease in the body breaks the microecological balance of the patient’s intestinal tract. During the process, bone metabolism will be regulated by the body’s overall “immune network-endocrine-nervous system”. Ultimately, this may aggravate inflammatory reactions and accelerate bone absorption\(^{13}\). In clinic, osteoporosis frequently arises in menopausal women with decreased levels of estrogen.

**Table III.** Changes in serum OPG and RANKL levels in the two groups of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>OPG (ng/mL)</th>
<th>RANKL (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (n = 25)</td>
<td>1.52 ± 0.31</td>
<td>82.69 ± 12.33</td>
</tr>
<tr>
<td>Observation group (n = 25)</td>
<td>4.25 ± 0.87</td>
<td>25.87 ± 6.36</td>
</tr>
<tr>
<td>( t )</td>
<td>14.779</td>
<td>20.478</td>
</tr>
<tr>
<td>( p )</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

**Table IV.** Changes in the levels of serum PICP and BMD in the two groups of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>PICP (μg/L)</th>
<th>BMD (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (n = 25)</td>
<td>2.87 ± 0.69</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>Observation group (n = 25)</td>
<td>15.66 ± 1.84</td>
<td>0.24 ± 0.08</td>
</tr>
<tr>
<td>( t )</td>
<td>3.079</td>
<td>0.500</td>
</tr>
<tr>
<td>( p )</td>
<td>0.002</td>
<td>0.310</td>
</tr>
</tbody>
</table>

**Table V.** Changes in serum BALP, TRACP and Ca levels in the two groups of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>BALP (U/L)</th>
<th>TRACP (U/L)</th>
<th>Ca (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (n = 25)</td>
<td>0.26 ± 0.03</td>
<td>31.05 ± 6.59</td>
<td>1.83 ± 0.26</td>
</tr>
<tr>
<td>Observation group (n = 25)</td>
<td>0.13 ± 0.02</td>
<td>50.33 ± 7.55</td>
<td>2.89 ± 0.30</td>
</tr>
<tr>
<td>( t )</td>
<td>18.028</td>
<td>9.619</td>
<td>13.351</td>
</tr>
<tr>
<td>( p )</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Figure 1. Correlation between osteoporosis and intestinal flora. A, Relationship between *Bifidobacterium* and intestinal flora changes. B, Relationship between *Escherichia coli* and intestinal flora changes. C, Relationship between *Lactobacillus* and intestinal flora changes.
In the present study, ovariectomized rats were classical research model rats with osteoporosis. Treatment supplemented with probiotics such as *Lactobacillus rhamnosus* to osteoporosis rats can markedly improve the BMD of rats. However, osteoporosis symptoms of rats supplemented with non-probiotics show no improvement. The results of this study suggested that the occurrence of osteoporosis in rats with inflammatory bowel disease was negatively correlated with the count of *Escherichia coli*, whereas it was positively related to the counts of *Lactobacillus* and *Bifidobacterium*. The above three representative bacteria were selected for research, of which most *Bifidobacterium* and *Lactobacillus* were considered as probiotics. Meanwhile, *Escherichia coli* was regarded as non-probiotics in most clinical studies. All these findings suggest that the imbalance of intestinal flora in rats exerts a crucial effect in the process of osteoporosis in rats with inflammatory bowel disease.

Currently, Ulukoylu et al. have manifested that RANKL is an OPG ligand that is capable of inducing osteoclast (OC) formation and efficaciously facilitating OC bone absorption *in vivo*. RANK can be expressed on the surface of OC precursors. Meanwhile, *Escherichia coli* was regarded as non-probiotics in most clinical studies. The above three representative bacteria were selected for research, of which most *Bifidobacterium* and *Lactobacillus* were considered as probiotics. Meanwhile, *Escherichia coli* was regarded as non-probiotics in most clinical studies. All these findings suggest that the imbalance of intestinal flora in rats exerts a crucial effect in the process of osteoporosis in rats with inflammatory bowel disease.

Conclusions

To sum up, osteoporosis in rats with inflammatory bowel disease has a negative association with the count of *Escherichia coli*, and positive correlations with the counts of *Lactobacillus* and *Bifidobacterium*. Treatment with probiotics can effectively alleviate osteoporosis symptoms in rats with inflammatory bowel disease by influencing the level of corresponding cytokines. The novelty of this study was that all our findings are of guiding significance in clinic to some extent.
Conflict of Interest
The Authors declare that they have no conflict of interests.

Acknowledgements
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References

1) HALMOS T, SUBA I. [Non-alcoholic fatty liver disease, as a component of the metabolic syndrome, and its causal correlations with other extrahepatic diseases]. Orv Hetil 2017; 158: 2051-2061.


