European Review for Medical and Pharmacological Sciences 2020; 24: 5697-5702

The relationship between intestinal flora changes and osteoporosis in rats with inflammatory bowel disease and the improvement effect of probiotics

Y. ZHONG[1], C. ZHENG[2], J.-H. ZHENG[3], S.-C. XU[4]

[1]Department of Orthopaedics, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China
[2]The First Clinical Medical College of Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China
[3]Department of Orthopaedics, Xianju People's Hospital, Xianju, Zhejiang, China
[4]Department of General Medicine, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China

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 tissues[1]. The incidence rate of osteoporosis in many clinical diseases of

Corresponding Author: Shanchun Xu, MM; e-mail: xu6452xu@126.com

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-

Table I. Culture counts of *Bifidobacteria*, *Lactobacillus* and *Escherichia coli* in the two groups of rats (lgCFU/g stools).

Group	*Escherichia coli*	*Lactobacillus*	*Bifidobacteria*
Bowel disease group (n = 50)	4.52 ± 0.39	5.13 ± 0.33	4.98 ± 0.59
Osteoporosis group (n = 50)	5.89 ± 0.28	4.29 ± 0.41	4.22 ± 0.37
t	20.178	11.286	7.717
p	< 0.001	< 0.001	< 0.001

tration (Ca) and procollagen type I carboxy-terminal propeptide (PICP) strictly according to the instructions of the kit bought from Shanghai Yiyan Biotechnology Co., Ltd. (Shanghai, China). Measurement of bone mineral density (BMD) level[10]: 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 ($\bar{x} \pm s$) 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 II. Relationship between the incidence of relevant osteoporosis in rats with inflammatory bowel disease and changes in intestinal flora.

Item	*r*	*p*
Escherichia coli	0.584	0.012
Lactobacillus	-0.693	< 0.001
Bifidobacteria	-0.656	< 0.001

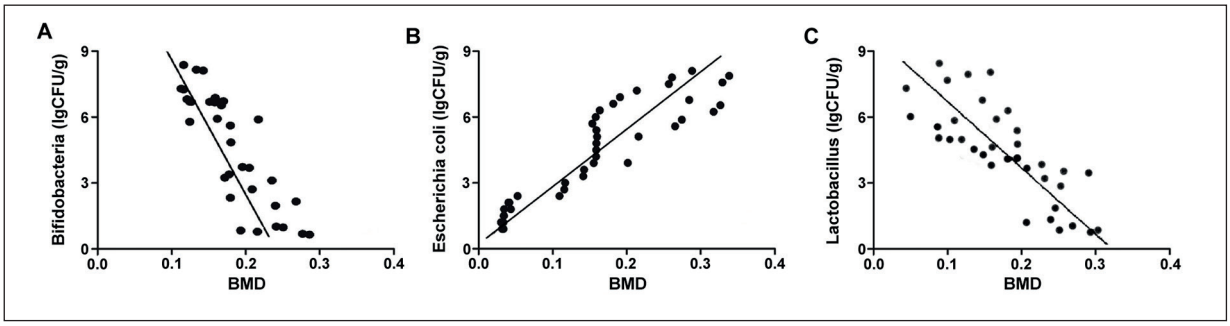

Figure 1. Correlation between osteoporosis and intestinal flora. **A,** Relationship between *Bifidobacterium* and intestinal flora changes. **B,** Relationship between *Escherichia coli* and intestinal flora changes. **C,** Relationship between *Lactobacillus* and intestinal flora changes.

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.

Group	OPG (ng/mL)	RANKL (pg/mL)
Control group (n = 25)	1.52 ± 0.31	82.69 ± 12.33
Observation group (n = 25)	4.25 ± 0.87	25.87 ± 6.36
t	14.779	20.478
p	< 0.001	< 0.001

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

Group	PICP (μg/L)	BMD (g/cm^2))
Control group (n = 25)	2.87 ± 0.69	0.23 ± 0.06
Observation group (n = 25)	15.66 ± 1.84	0.24 ± 0.08
t	3.079	0.500
p	0.002	0.310

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

Group	BALP (U/L)	TRACP (U/L)	Ca (mmol/L)
Control group (n = 25)	0.26 ± 0.03	31.05 ± 6.59	1.83 ± 0.26
Observation group (n = 25)	0.13 ± 0.02	50.33 ± 7.55	2.89 ±0.30
t	18.028	9.619	13.351
p	< 0.001	< 0.001	< 0.001

Table VI. Changes in the levels of serum IL-6, TNF-α and IFN-γ in the two groups of rats (pg/mL).

Group	IL-6	TNF-α	IFN-γ
Control group (n = 25)	105.31 ± 15.66	36.52 ± 5.39	13.58 ± 3.62
Observation group (n = 25)	20.51 ± 3.25	13.62 ± 4.98	5.40 ± 2.55
t	26.510	15.603	9.237
p	< 0.001	< 0.001	< 0.001

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[14]. 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 Bifidobacteria*. The above three representative bacteria were selected for research, of which most *Bifidobacteria* 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[15].

Currently, Ulukoylu et al[16] 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, it combines with RANKL on the surface of stromal cells and osteoblasts, thereby triggering OC maturation and differentiation. OPG mainly neutralizes or combines with RANKL binding to stromal cells and soluble RANKL[17]. Therefore, the changes in the levels of serum RANKL and OPG levels in rats can influence bone resorption and formation. The results of this study implied that probiotic therapy for rats with inflammatory bowel disease complicated with osteoporosis could alleviate osteoporosis symptoms by increasing OPG level, decreasing RANKL level, and enhancing bone absorption and formation. PICP, a vital member of the collagen family, effectively maintains the biomechanical properties of rat bones and the integrity of bone structures. Moreover, bone turnover and the synthesis rate of type I collagen can be clearly reflected by its serum content[18]. Shen et al[19] have illustrated that serum PICP can also effectively reflect bone formation level and bone cell function. In this study, we found that the level of serum PICP in rats was significantly upregulated after treatment with probiotics. This is probably due to the reason that probiotics can alleviate osteoporosis symptoms by improving intestinal nutrients, antagonizing intestinal pathogenic bacteria and affecting bone absorption. It can be concluded from this study that probiotics elevates the level of serum inflammatory cytokines in rats, so as to improve osteoporosis. The primary reason may be that IL-6 functions mainly in the early stage of OC and stimulates the division and proliferation of OC precursors. In addition, it is able to synergize with other hormones and cytokines to modulate bone absorption and OC formation. TNF-α is a bone absorption promoter, which suppresses bone formation and OC apoptosis. Besides, TNF-α is an "upstream" cytokine that acts on OC. It has been indicated to induce the secretion of relevant cytokines that can stimulate pre-OC differentiation in hematopoietic stem cells[20]. Under such conditions, a minor change in TNF-α can result in a dramatic change in OC formation, thus indirectly inhibiting OC formation.

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

The Project was supported by Zhejiang Provincial Natural Science Foundation of China (Grant No. LQ18H270003).

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.
2) Seyfizadeh N, Seyfizadeh N, Negahdar H, Hosseini SR, Nooreddini H, Parsian H. ABO blood group and prevalence of osteoporosis and osteopenia in the elderly population: an Amirkola Health and Ageing Project (AHAP)-Based Study. J Clin Densitom 2018; 21: 200-204.
3) Huang F, Zhao S, Qiu M, Li Y, Zhan X, Wu C, Shi C, Sun W, Chen G, Xu Y. Acupuncture for primary osteoporosis: a network meta-analysis of randomized controlled trials protocol. Medicine (Baltimore) 2019; 98: e15108.
4) Rollo G, Pichierri P, Marsilio A, Filipponi M, Bisaccia M, Meccariello L. The challenge of nonunion after osteosynthesis of the clavicle: is it a biomechanical or infection problem? Clin Cases Miner Bone Metab 2017; 14: 372-378.
5) Romagnoli C, Zonefrati R, Galli G, Aldinucci A, Nuti N, Martelli FS, Tonelli P, Tanini A, Brandi ML. The effect of strontium chloride on human periodontal ligament stem cells. Clin Cases Miner Bone Metab 2017; 14: 283-293.
6) Lambert M, Thybo CB, Lykkeboe S, Rasmussen LM, Frette X, Christensen LP, Jeppesen PB. Combined bioavailable isoflavones and probiotics improve bone status and estrogen metabolism in postmenopausal osteopenic women: a randomized controlled trial. Am J Clin Nutr 2017; 106: 909-920.
7) Wilkins T, Sequoia J. Probiotics for gastrointestinal conditions: a summary of the evidence. Am Fam Physician 2017; 96: 170-178.
8) Takeuchi K, Komatsu Y, Nakamori Y, Kotani T. A rat model of ischemic enteritis: pathogenic importance of enterobacteria, iNOS/NO, and COX-2/PGE2. Curr Pharm Des 2017; 23: 4048-4056.
9) Liu H, Xiong Y, Wang H, Yang L, Wang C, Liu X, Wu Z, Li X, Ou L, Zhang R, Zhu X. Effects of water extract from epimedium on neuropeptide signaling in an ovariectomized osteoporosis rat model. J Ethnopharmacol 2018; 221: 126-136.
10) Li X, Ominsky MS, Villasenor KS, Niu QT, Asuncion FJ, Xia X, Grisanti M, Wronski TJ, Simonet WS, Ke HZ. Sclerostin antibody reverses bone loss by increasing bone formation and decreasing bone resorption in a rat model of male osteoporosis. Endocrinology 2018; 159: 260-271.
11) Ma Q, Li Y, Li P, Wang M, Wang J, Tang Z, Wang T, Luo L, Wang C, Wang T, Zhao B. Research progress in the relationship between type 2 diabetes mellitus and intestinal flora. Biomed Pharmacother 2019; 117: 109138.
12) Huyut Z, Bakan N, Yildirim S, Alp HH. Effects of the phosphodiesterase-5 (PDE-5) inhibitors, Avanafil and Zaprinast, on bone remodeling and oxidative damage in a rat model of glucocorticoid-induced osteoporosis. Med Sci Monit Basic Res 2018; 24: 47-58.
13) Brennan O, Sweeney J, O'Meara B, Widaa A, Bonnier F, Byrne HJ, O'Gorman DM, O'Brien FJ. A natural, calcium-rich marine multi-mineral complex preserves bone structure, composition and strength in an ovariectomised rat model of osteoporosis. Calcif Tissue Int 2017; 101: 445-455.
14) Britton RA, Irwin R, Quach D, Schaefer L, Zhang J, Lee T, Parameswaran N, McCabe LR. Probiotic L. reuteri treatment prevents bone loss in a menopausal ovariectomized mouse model. J Cell Physiol 2014; 229: 1822-1830.
15) Zhang H, Shi X, Wang L, Li X, Zheng C, Gao B, Xu X, Lin X, Wang J, Lin Y, Shi J, Huang Q, Luo Z, Yang L. Intramembranous ossification and endochondral ossification are impaired differently between glucocorticoid-induced osteoporosis and estrogen deficiency-induced osteoporosis. Sci Rep 2018; 8: 3867.
16) Ulukoylu E, Karatas E, Albayrak M, Bayir Y. Effect of calcium hydroxide alone or in combination with ibuprofen and ciprofloxacin on nuclear factor kappa B ligand and osteoprotegerin level in periapical lesions: a randomized controlled clinical study. J Endod 2019; 45: 1489-1495.
17) Witkowska-Sedek E, Ruminska M, Stelmaszczyk-Emmel A, Sobol M, Demkow U, Pyrzak B. Osteoprotegerin, receptor activator of nuclear factor kappa B ligand, and growth hormone/insulin-like growth factor-1 axis in children with growth hormone deficiency. Adv Exp Med Biol 2018; 1116: 63-73.
18) Soleyman-Jahi S, Yousefian A, Maheronnaghsh R, Shokraneh F, Zadegan SA, Soltani A, Hosseini SM, Vaccaro AR, Rahimi-Movaghar V. Evidence-based prevention and treatment of osteoporosis after spinal cord injury: a systematic review. Eur Spine J 2018; 27: 1798-1814.
19) Shen GY, Ren H, Tang JJ, Qiu T, Zhang ZD, Zhao WH, Yu X, Huang JJ, Liang, Yao ZS, Yang ZD, Jiang XB. Effect of osteoporosis induced by ovariectomy on vertebral bone defect/fracture in rat. Oncotarget 2017; 8: 73559-73567.
20) Fredoni M, Ghatrehsamani M, Abdollahifar MA, Bayat S, Bayat M. Evaluation of the effects of photobiomodulation on vertebras in two rat models of experimental osteoporosis. Lasers Med Sci 2017; 32: 1545-1560.