ABSTRACT

The Ca\textsuperscript{2+}-selective epithelial channel TRPV5 plays a significant role in renal calcium reabsorption and improving osteoporosis (OP). In this study, we investigated the mechanisms of yak milk on osteoporosis mice in TRPV5-mediated Ca\textsuperscript{2+} reabsorption in the kidney. We observed that treatment of OP mice with yak milk reconstructed bone homeostasis demonstrated by increasing the levels of OPG as well as decreasing the levels of TRAP and ALP in serum. Additionally, yak milk reduced the level of parathyroid hormone (PTH) and elevated 1,25-(OH)\textsubscript{2}D\textsubscript{3} and calcitonin (CT), and inhibited the excretion of Ca/Cr and Pi/Cr in OP mice, which explained by regulating hormone levels and thus enhance the renal Ca\textsuperscript{2+} reabsorption. Further analysis exhibited that yak milk upregulated the expression of TRPV5 protein and mRNA as well as calbindin-D\textsubscript{28k} in OP mice kidneys. Overall, these outcomes demonstrate that yak milk enhances renal Ca\textsuperscript{2+} reabsorption through the TRPV5 pathway synergistically with calbindin-D\textsubscript{28k}, thus ameliorating OP mice. This provides a new perspective for yak milk as a nutritional supplement to prevent osteoporosis.

Key words: yak milk, calcium, reabsorption, osteoporosis, TRPV5

INTRODUCTION

Osteoporosis (OP) is a disease that seriously endangers human health. It is characterized by bone mass loss and deterioration of bone microstructure (Tu et al., 2015). Calcium is the main mineral in the bones, providing bone strength and maintaining calcium homeostasis (Fischer et al., 2018). Therefore, calcium deficiency is one of the main risk factors for osteoporosis. Ca\textsuperscript{2+} plays a crucial role in our bodies. It serves as both the intracellular messenger and the primary constituent of bone. In general, a complex set of transport processes controlled by proteins in the pathway, hormones, and physiological factors mediate Ca\textsuperscript{2+} transport.

The kidney plays a significant role in maintaining Ca\textsuperscript{2+} balance by regulating Ca\textsuperscript{2+} excretion in the body. The filtered Ca\textsuperscript{2+} is widely absorbed as it passes through the various renal tubules. Transient receptor potential channel proteins are a diverse and large family of proteins involved in many life processes (Hoenderop et al., 2003a). Vanilloid (TRPV) is one of its many subfamilies, including TRPV5 and TRPV6, which are the molecular gatekeepers facilitating Ca\textsuperscript{2+} influx in the kidney, small intestine, and bone. TRPV5 is mainly expressed in the kidney, which is thought to maintain Ca\textsuperscript{2+} in the body by promoting renal Ca\textsuperscript{2+} reabsorption (van der Wijst et al., 2019a). Simultaneously, calbindins are a vitamin D-dependent Ca\textsuperscript{2+}-binding protein. Calbindin-D\textsubscript{28k} is widely recognized as a key component of Ca\textsuperscript{2+} processing in the body and is essential for renal Ca\textsuperscript{2+} transport (Lambers et al., 2006). Parathyroid hormone (PTH), 1,25-dihydroxyvitamin D\textsubscript{3} (1,25-(OH)\textsubscript{2}D\textsubscript{3}), and calcitonin (CT) regulate total body Ca\textsuperscript{2+} homeostasis. 1,25-(OH)\textsubscript{2}D\textsubscript{3} promotes Ca\textsuperscript{2+} absorption. These 3 hormones interact with each other to maintain calcium homeostasis (Suzuki et al., 2008).

Currently, researchers have developed a variety of agents for the treatment of OP, such as bisphosphonates and calcitonin (Muñoz-Torres et al., 2004), which can inhibit osteoclast activity and enhance bone formation. However, these substances exhibit some side effects, including a high risk of osteonecrosis and fracture of the jaw (Hong et al., 2010), as well as symptoms such as acid reflux and mucosal erosion (Papapetrou, 2009). Therefore, it is necessary to find a potential natural product that can effectively and safely prevent OP. Numerous studies have reported that many natural products can prevent osteoporosis, such as tea polysaccharides (Xu et al., 2018) and rhodopsin phenolic...
extracts (Zhuang et al., 2020). In addition, estrogen can improve osteoporosis in ovariectomized rats by regulating renal calcium reabsorption (van Abel et al., 2002), and specific functional proteins such as hPThrP1-34 and 1-84 can also improve osteoporosis by enhancing renal calcium reabsorption (Wang et al., 2014).

Milk and dairy products are excellent carriers of nutrition fortification due to their bioactive components and thus may reduce the risk of osteoporosis (Ilesanmi-Oyelere and Kruger, 2020). In current studies, lactose and probiotics in milk and dairy products have been found to promote calcium absorption (Jafarnejad et al., 2017; Nath et al., 2018), and basic protein and acidic protein fractions and lactoferrin in milk have been shown to have significant effects on osteoporosis (Krugger et al., 2006; Uenishi et al., 2007; Hou et al., 2012). However, our previous studies reveal that Pamir yak milk has excellent nutritional content. Compared with goat and cow milk, it has higher contents of fat (4.63%), protein (4.30%), total solids (14.84%), lactose (5.21%), and minerals such as Ca (152 ± 0.01 mg/100g). Yak milk is rich in essential amino acids, which account for 48% of the total amino acids (Mamet et al., 2023). Meanwhile, yak milk can also improve bone mineral density and microstructure in OP mice via increased intestinal calcium absorption (Li et al., 2022). To the best of our knowledge, results of administration yak milk on OP in TRPV5-mediated Ca\(^2+\) reabsorption in the kidney have yet to be reported. Therefore, the objective of the present study was to investigate how yak milk contributes to the regulation of TRPV5 channel activity in renal calcium in C57BL/6J mice with retinoic acid (RA)-induced osteoporosis.

**MATERIALS AND METHODS**

**Chemicals and Materials**

Alendronate sodium trihydrate (B25874) and RA (B21287) were procured from Shanghai Yuanye Biotechnology Co. Ltd. Paraformaldehyde universal tissue fixative (4%; BL539A) was obtained from Biosharp. An alkaline phosphatase (ALP) kit (A059-2-2) and phosphorus (Pi) kit (C006-1-1) were purchased from Nanjing Jiancheng Bioengineering Institute. A calcium content color detection kit (SI063AS) and antitartrate acid phosphatase (TRAP) detection kit (P0332) were purchased from Beyotime Biotechnology. Mouse 1,25-dihydroxyvitamin D\(_3\) (1,25-(OH)\(_2\)D\(_3\)) ELISA kit (MU30655), mouse PTH ELISA kit (MU30056) and mouse CT ELISA kit (MU30989) were provided by Bioswamp. A mouse osteoprotegerin (OPG) ELISA kit (CSB-E04693m) was procured from CUSABIO. The anti-TRPV5 antibody (M03218), anticalbindin-D\(_{28k}\) antibody (PB9045) and hypersensitive chemiluminescence kit (AR1174) were procured from BOSTER Biological Technology Co. Ltd. Goat anti-rabbit IgG heavy and light chains (horseradish peroxidase, HRP; ab6721) was purchased from Abcam. TransZol up plus RNA kit (ER501-01) and green Quantitative real-time PCR (qPCR) superMix (AQ101-01) were provided by TransGen Biotech Co. Ltd.

**Sample Collection and Preparation**

In mid-July 2021, milk samples from 120 Pamir yak individuals were collected from Akqi County, Kizilsu Kyrgyz Autonomous Prefecture, Xinjiang Uygur Autonomous Region, China. All yak milk samples were mixed (28 kg) and pasteurized before being transported to the laboratory on dry ice and stored in a −80°C freezer for subsequent use. Yak milk was vacuum freeze-dried into powder using an Alpha 1-2 LDplus vacuum freeze dryer (Christ) for animal experiments.

**Animal Experiment**

Experiments were conducted in accordance with current legislation regarding animal experiments in the Animal Experiment Center of Xinjiang Medical University (Urumqi, China; Licensed ID: SCXK 2018-0002). C57BL/6J mice (9 mo old, male, weight 33.18 ± 0.80 g) of specific-pathogen-free grade were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Licensed ID: SCXK2021-0011). Laboratory mouse maintenance feed (1010088) was obtained from the Animal Experiment Center of Xinjiang Medical University, which complies with Chinese national standard GB 14924.1 (General Quality Standards for Compound Feeds for Experimental Animals).

The mice were raised as described previously (Li et al., 2022). Briefly, mice were continuously gavaged with RA (70 mg/kg per day) for 2 weeks to establish osteoporosis model (n = 32), and the control group (n = 8) was given the same amount of vegetable oil. After 2 weeks, the modeling mice were randomly divided into 4 groups, namely the model group, low-dose yak milk group (L-YM, 1,000 mg/kg per day), high-dose yak milk group (H-YM, 2,000 mg/kg per day), and alendronate sodium group (ALN, 1 mg/kg per day). The L-YM (yak milk powder:water = 1:10) and H-YM (yak milk powder:water = 1:5) groups were fed via gavage once per day. The control group and model group were given the same amount of distilled water. The mice were kept at a temperature of 20 ± 3°C and a humidity of 60 ± 5% under 12-h light/dark conditions.
and had free access to standard feed (laboratory mouse maintenance feed) and distilled water. There was no significant difference in the amount of food intake among the groups (data not shown, $P < 0.05$). After 6 weeks of treatment, mice were slaughtered by cervical dislocation after overnight feeding.

**Serum and Urine Parameters**

Before the mice were slaughtered, 24-h urine was collected from the mice by the metabolic cage method and placed in a $-80^\circ$C refrigerator. Mice were euthanized with sodium pentobarbital 50 mg/kg BW. Eyeballs were collected for blood collection. The blood was collected in a 1.5-mL centrifuge tube and coagulated at room temperature, followed by centrifuging at 1,200 × $g$ for 10 min at 4°C. The supernatant was collected in a new centrifuge tube and placed at $-80^\circ$C for analysis. Alkaline phosphatase and Pi were detected by ALP kit (A059-2-2) and Pi kit (C006-1-1). A calcium content color detection kit (S1063S) and a TRAP detection kit (P0332) were used to detect Ca$^{2+}$ and TRAP. Mouse 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) ELISA kit (MU30655), mouse PTH ELISA kit (MU30056), mouse CT ELISA kit (MU30989), and mouse OPG ELISA kit (CSB-E04693m) were used to measure 1,25-(OH)$_2$D$_3$, PTH, CT, and OPG.

**Organ Index Measurement**

The mice were weighed before they were slaughtered. After the mice were slaughtered, the left kidney, right kidney, liver, and heart were quickly taken out and weighed. The organ index of each organ of the mice was calculated using the following formula:

\[
\text{Organ index (mg/g)} = \frac{\text{organ weight (mg)}}{\text{BW (g)}}.
\]

**Immunohistochemistry**

Immunohistochemistry was performed using the method described by Bolognesi et al. (2017) with minor modifications. The kidney samples from mice were fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. The specimens were cut into 5-μm sections and then deparaffinized with xylene and alcohol (100%, 95%, 75%, 50%, respectively). Sections of mice kidneys were subjected to antigen retrieval with citrate and blocked with goat serum. Primary antibody anti-TRPV5 and anticalbindin-D$_{28k}$ were incubated overnight at 4°C, followed by 1 h of incubation with anti-rabbit IgG-HRP. 3,3′-diaminobenzidine hydrochloride was used for color development. After counter-staining with hematoxylin, the sections were dehydrated and permeabilized. The sections were observed under a Nikon Optical Light Microscope (Eclipse Ci-S, Nikon) with a 40× objective, and the images were captured.

**Western Blot**

Proteins from mice kidney cortex were introduced to 10% SDS-PAGE and were subsequently electrobotted onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% skim milk-TBS-Tween20 for 1 h at 37°C and incubated overnight at 4°C with anti-TRPV5 antibody, anticalbindin-D$_{28k}$ antibody and β-actin antibody (diluted 1:1,000 in PBS). After incubating these proteins with HRP-conjugated anti-rabbit IgG, their expression was observed with a chemiluminescence kit. Finally, the membrane was viewed on Las 4000 (FVJIFILM corporation). The density of the bands was determined using Image J 1.8.0 (National Institutes of Health).

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was isolated from the renal cortex using the TransZol reagent. The template DNA was obtained by reverse transcription of the total RNA of the sample. Real-time qPCR was conducted using a real-time RT-PCR assay in a final volume of 10 μL according to the manufacturer’s protocol. Results were analyzed using Applied Biosystems 7500 Fast System SDS software (ThermoFisher). The expression levels of mRNAs were

<table>
<thead>
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<th>Direction</th>
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<th>Length (bp)</th>
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<tr>
<td></td>
<td>Rev</td>
<td>CCAGTTGGTAACAAATGCACATG</td>
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calculated by the standard $2^{-\Delta\Delta Ct}$ method with $\beta$-actin as internal control. The resulting calibrated normalized relative quantities were used for statistical analysis. The primer pairs used are shown in Table 1.

**Statistical Analysis**

All data were displayed as mean ± standard deviation. Data analysis was performed using IBM SPSS Statistics 21.0 software (SPSS Inc.), and bar graphs were drawn with GraphPad Prism 8.0.2 (GraphPad Software). Statistical comparisons were assessed by one-way ANOVA and the least significant difference test. A $P < 0.05$ value was considered to be statistically significant.

**RESULTS**

**Effects of Yak Milk on Organ Index in OP Mice**

To investigate the effect of yak milk on osteoporosis, we measured body weight changes and organ indices in OP mice (Figure 1). During the modeling period, the weight of mice in each administration group generally decreased, which may result from the toxic effects of excess RA, and then gradually increased after RA treatment was discontinued (Figure 1A). It is worth noting that although there was no significant difference in body weight between groups after 6 weeks of treatment, the yak milk treatment group showed a good effect of body weight recovery at the initial stage of
treatment. This phenomenon may be related to the fact that yak milk is rich in nutrients.

However, the organ index analysis showed that yak milk could reverse the changes in organ index caused by RA (Figure 1B). Compared with the control group, the kidney, liver, and heart weights of the model group were significantly decreased \((P < 0.05)\), where the weight of indexes was increased to varying degrees after yak milk treatment. The yak milk treatment group could significantly increase the organ weight indexes of the liver and kidney of mice \((P < 0.05)\) as compared with the model group. Otherwise, the weight of the heart in the yak milk treatment group was also slightly increased, but the difference was not significant. This indicates that yak milk alleviated the liver and kidney damage caused by RA in OP mice.

**Effects of Yak Milk on Bone Metabolism in OP Mice**

Bone turnover markers can be used to evaluate the effectiveness of treatment for osteoporosis and are a useful aid in monitoring osteoporosis treatment (Greenblatt et al., 2017). Antititrarate acid phosphatase, ALP, and OPG are typical markers of bone turnover. The data in Figure 2A and 2B showed that the activities of TRAP and ALP in the RA-induced model group were significantly increased \((19.80 \pm 0.89 \text{ and } 153.62 \pm 11.92 \text{ U/L, respectively}; \ P < 0.05)\), compared with the control group. After yak milk treatment, TRAP levels in L-YM and H-YM groups were decreased to 15.71 and 10.39 U/L, and ALP levels were also reduced to 121.12 and 109.18 U/L, respectively. The ALN group showed the same trend. These indicate that yak milk significantly improved the effect on RA-induced OP.

**Effect of Yak Milk on Calcium Homeostasis in OP Mice**

To investigate the effects of yak milk on calcium homeostatic and renal calcium reabsorption, the levels of serum calcium, phosphorus, 1,25-(OH)\(_2\)D\(_3\), CT, and PTH, as well as urine Ca/Cr and Pi/Cr were detected (Figure 3). The results showed that the serum calcium and the urine Ca/Cr and Pi/Cr in model group were significantly increased, whereas the serum phosphorus was reduced compared with the control group \((P < 0.05)\). However, both the urine Ca/Cr and the Pi/Cr were decreased by yak milk administration (L-YM, H-YM, and ALN groups). Additionally, the serum 1,25-(OH)\(_2\)D\(_3\) and CT levels in model group were decreased, while the serum 1,25-(OH)\(_2\)D\(_3\) and CT levels were increased in L-YM, H-YM, and ALN groups. The serum PTH levels were also reduced in a dose-dependent manner in yak milk treatment group. These outcomes imply that yak milk can maintain calcium homeostasis in RA-induced OP mice, which may be achieved by regulating the related hormone levels as well as the reabsorption of Ca\(^{2+}\).

**Immunohistochemistry of the Kidney**

We investigated the expression levels of TRPV5 and calbindin-D\(_{28k}\) in the mice kidney to explore the in-
ternal mechanism of yak milk influencing OP (Figure 4A). As shown in Figure 4B and C, the model group had fewer TRPV5 and calbindin-D28k positive cells, and the average absorbance was significantly decreased ($P < 0.05$) compared with the control group. Following treatment with yak milk (L-YM and H-YM) and ALN, enhanced expression of TRPV5 and calbindin-D28k. This means that the protein abundance of TRPV5 and calbindin-D28k in yak milk treatment group was significantly increased ($P < 0.05$) in a dose-dependent manner compared with the model group. This observed that yak milk affects renal Ca$^{2+}$ transports dependent on the TRPV5 channel, which synergistic calbindin-D28k.

**Effects of Yak Milk on the Expression of TRPV5 and Calbindin-D28k**

To clarify the mechanism of renal calcium reabsorption by yak milk, the expression levels of TRPV5 and calbindin-D28k protein were tested and β-actin was used as internal reference. As shown in Figure 5A, B,
and C, the expression of TRPV5 and calbindin-D28k decreased by 38.14% and 24.23% in OP mice after RA induction. However, after administration of yak milk, the expression of TRPV5 and calbindin-D28k markedly increased, especially H-YM group (75.74% and 64.56%, respectively). These data indicated that yak milk could significantly increase the expression of TRPV5 and calbindin-D28k in the kidney of OP mice \((P < 0.05)\), which was in line with the results of immunohistochemistry.

**Effects of Yak Milk on TRPV5 and Calbindin-D28k mRNA**

To further investigate the effect of yak milk on the expression of TRPV5 and calbindin-D28k in the kidney of OP mice, qPCR was performed to quantitatively detect by real-time fluorescence. As can be seen in Figure 6A and B, compared with the control group, the mRNA expressions of TRPV5 and calbindin-D28k in the model group were significantly decreased (33.09% and 36.78%, \(P < 0.05\)). The yak milk treatment group reversed this trend. TRPV5 and calbindin-D28k mRNA were increased to 89.59% and 91.61% in L-YM group, respectively. Likewise, TRPV5 mRNA increased to 169.69% and calbindin-D28k mRNA increased to 142.60% in H-YM group. The results revealed that yak milk significantly increased the mRNA expression levels of TRPV5 and calbindin-D28k in the kidney of OP mice in a dose-dependent manner \((P < 0.05)\).

**DISCUSSION**

Consumption of milk and dairy products is beneficial for human health; some researchers have reported that women with low milk intake during childhood and adolescence have less bone mass in adulthood and greater risk of fracture (Kalkwarf et al., 2003). Low-fat milk with the greatest proportion of protein intake has been shown to be beneficial to the skeleton (Mangano et al., 2015). However, unlike other types of milk, yak milk contains high levels of protein, many unsaturated fatty acids, minerals, and lactose (Singh et al., 2023). Our research found that yak milk improves bone loss in OP mice. But the effect of yak milk on the renal Ca\(^{2+}\) resorption of OP mice remains unclear.

In this study, the RA-induced OP model has been used, and the control group, a model group, L-YM, and H-YM were established, whereas OP mice treated
with ALN were used as a positive control. We demonstrated that yak milk, especially H-YM, could modify the organ indexes of the kidney, femur, tibia, heart, and liver, and recover the weight loss caused by RA in contrast with the model group (Figure 1), which may be related to yak milk benefits for damage of the organ by RA, consistent with the results of Wang et al. (2019). Additionally, TRAP can promote bone resorption, and ALP can inhibit osteoclast. OPG can also inhibit osteoclast differentiation by blocking RANKL-RANK interaction, which is essential for regulating the balance between bone formation and resorption (McClung, 2006). In the current study, we found that TRAP and ALP levels in serum of OP mice were decreased after yak milk treatment, while serum OPG levels were significantly increased (∗∗∗P < 0.05), which contributes to enhanced bone formation and bone homeostasis. Yak milk worked similarly to ALN (Figure 2). Interestingly, Liu et al. claimed that Rehmanniae Radix Preparata rebuilt bone homeostasis demonstrated by increasing the levels of OPG as well as decreasing the levels of TRAP, RANKL, and ALP in serum (Liu et al., 2019). Therefore, these observations exhibited that yak milk may inhibit RA-induced bone resorption and osteoclast differentiation in OP mice through the regulation of bone homeostasis.

The maintenance of Ca^{2+} balance in vivo is essential for many important physiological functions, including bone formation, resorption, and muscle contraction, and is tightly controlled by the kidney and bone (Hoenderop et al., 2005). Many methods have been used to study renal Ca^{2+} uptakes, such as metabolic homeostasis, puncture, and characterization of transporters (Bindels, 1993). Parathyroid hormone, 1,25-(OH)_{2}D_{3}, and CT can regulate systemic calcium homeostasis. Simultaneously PTH can stimulate bone resorption and release Ca^{2+} from the bone. Furthermore, 1,25-(OH)_{2}D_{3} is believed to induce renal Ca^{2+} reabsorption and stimulate the small intestine to increase dietary Ca^{2+} absorption, and CT can also promote renal Ca^{2+} reabsorption, enhance bone formation, and promote bone mineralization (Hoenderop et al., 2003a, 2003b).
In this study, the observed levels of PTH were decreased and 1,25-(OH)₂D₃ and CT were increased in OP mice treated with yak milk compared with those in the model group (Figure 3). These results also demonstrate that yak milk could reduce osteoporosis in mice by improving renal calcium reabsorption, thus enhancing bone formation and inhibiting bone resorption through hormonal regulation. Moreover, serum calcium and phosphorus levels reflect bone metabolism, and increased urine Ca/Cr and Pi/Cr excretion indicate increased bone resorption and decreased renal Ca²⁺ and Pi reabsorption (Jones et al., 2010; Blaine et al., 2015). Evidence has shown that OP can increase blood calcium, decrease blood phosphorus (El-Baz et al., 2019), and promote a high probability of hypercalciuria (El-Husseini et al., 2017). Under a normal diet, serum calcium returned to normal, and serum phosphorus value was raised after supplementation of yak milk, but it effectively inhibited Ca/Cr and Pi/Cr excretion (Figure 3). These obtained data also suggest that yak milk may inhibit the loss of urinary calcium and phosphorus in OP mice via strengthening renal reabsorption function and inhibiting bone resorption. However, the pathological process of osteoporosis is affected by gut microbes. Yak milk not only improves osteoporosis by inhibiting bone resorption and osteoclast differentiation, but also enhances calcium absorption through the production of specific metabolites such as short-chain fatty acids (Wallace et al., 2017; Ding et al., 2020). However, yak milk is a complex food matrix, with α-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid components, which may modify gut microbiota composition and metabolism. The role of yak milk compounds on microbiota composition also needs to be investigated to fully appreciate the role of yak milk in the regulatory effect of gut microbes on osteoporosis.

TRPV5 has confirmed that a Ca²⁺ permeant ion channel is identified as a result of increasing Ca²⁺ uptake activity in the kidney. Calbindin-D₂₈k colocalizes with the epithelial Ca²⁺ channel TRPV5, which makes up the apical entry step in the renal Ca²⁺-transporting mechanism (Na and Peng, 2014; Huang et al., 2019; van der Wijst et al., 2019b). In addition, the importance of TRPV5 in Ca²⁺ homeostasis is demonstrated in TRPV5 knockout mice (TRPV5⁻/⁻), which exhibited high urine Pi, Ca²⁺ excretion, and bone abnormalities, and low calbindin-D₂₈k expression (Hoenderop et al., 2003b; Moor and Bonny, 2016). Conversely, in calbindin-D₂₈k⁻/⁻ mice, TRPV5 protein abundance is not affected (Nijenhuis et al., 2005). However, in our study, the yak milk played a significant role in renal Ca²⁺ reabsorption in OP mice, which is explained by immunohistochemistry analysis. The TRPV5 and calbindin-D₂₈k protein abundance in the kidney was significantly increased (P < 0.05, Figure 4). This difference may be responsible for yak milk effect on renal Ca²⁺ reabsorption via active Ca²⁺ transporters such as TRPV5 and calbindin-D₂₈k.

With regard to the mechanisms underlying the yak milk facilitation of Ca²⁺ reabsorption, we obtained that
renal TRPV5 and calbindin-D$_{28k}$ protein levels were significantly elevated in OP mice ($P < 0.05$, Figure 5). Furthermore, qPCR assay clearly exhibited that yak milk upregulates TRPV5 mRNA (2.53-fold) as well as calbindin-D$_{28k}$ (2.25-fold) in OP mice kidneys. These results strongly suggest that yak milk promotes renal Ca$^{2+}$ reabsorption through TRPV5 pathway colocalizes with calbindin-D$_{28k}$, thus ameliorating OP in mice. Interestingly, more recent research has confirmed that glucocorticoids can be used to alleviate osteoporosis by raising the abundance of TRPV5 mRNA (Dittmer et al., 2021), and menthol can raise TRPV5 mRNA to enhance the level of calcium absorption in sheep (Geiger et al., 2021). Furthermore, Wang et al. demonstrated that parathyroid hormone related protein (PTHrP) could enhance the level of calcium absorption in sheep (Geiger et al., 2021). Furthermore, Wang et al. demonstrated that parathyroid hormone related protein (PTHrP) could also treat osteoporosis by promoting renal calcium reabsorption by elevating the expression of TRPV5 and calbindin-D$_{28k}$ (Wang et al., 2014). These conclusions are consistent with our research results.

In summary, yak milk can inhibit RA-induced bone resorption through hormonal regulation. Similarly, it can also inhibit the loss of urinary calcium and phosphorus by enhancing renal reabsorption function. These effects may be related to the facilitation of renal Ca$^{2+}$ reabsorption in OP mice through active TRPV5 and calbindin-D$_{28k}$. Our results also provide a new perspective for the application of yak milk to improve Ca$^{2+}$ absorption function, and attenuate OP. Further study is necessary to interpretation of the importance of gut microbiota and their metabolites in OP, especially yak milk active components. There are some limitations to this research. The specific interpretation of how yak milk improves osteoporosis compares with bovine milk or other mammalian milks need to be further verified.

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