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Heshouwu (*Polygonum multiflorum* Thunb.) Extract Attenuates Bone Loss in Diabetic Mice.

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ABSTRACT: This study investigated the effects and mechanism of Heshouwu (*Polygonum multiflorum* Thunb.) water extract (HSW) on diabetes-related bone loss in mice. HSW was orally administered (300 mg/kg body weight) to high-fat diet and streptozotocin-induced diabetic mice for 10 weeks. HSW significantly alleviated mouse body weight loss and hyperglycemia compared with the control group, and elevated serum levels of insulin, osteocalcin, and bone-alkaline phosphatase. HSW supplementation also significantly increased the bone volume/tissue volume ratio and trabecular thickness and number, and decreased the bone surface/bone volume ratio and trabecular structure model index in the femur and tibia. Moreover, HSW significantly increased femoral bone mineral density. In addition, HSW down-regulated osteoclastogenic genes, such as nuclear factor of activated T-cells, cytoplasmic 1 and tartrate-resistant acid phosphatase 5 (*TRAP*), in both the femur and tibia tissue, and reduced serum TRAP level compare to those of control mice. These results indicate that HSW might relieve diabetes-related bone disorders through regulating osteoclast-related genes, suggesting HSW may be used as a preventive agent for diabetes-induced bone loss.

Keywords: bone microarchitecture, diabetes, Heshouwu, *Polygonum multiflorum* Thunb., osteoporosis

INTRODUCTION

Diabetes mellitus (DM) is a common progressive metabolic disease that may lead to complications such as retinopathy, neuropathy, nephropathy, cardiovascular disease, and osteoporosis. Diabetic complications are major public health problems and are increasing in prevalence worldwide (Abdulameer et al., 2012). Fragility fractures have increasingly been recognized as a complication of both type 1 and 2 diabetes mellites (T1DM and T2DM, respectively) (Ferrari et al., 2018), and are the leading cause of morbidity and mortality in older adults with diabetes (Cauley, 2013). Low bone mineral density (BMD) is a risk factor of fragility fractures; BMD has been shown to decrease in T1DM, and to both decrease and increase in T2DM as compared healthy individuals (Napoli et al., 2017; Raška et al., 2017). Moreover, anti-diabetic medications, such as thiazolidinediones and sodium glucose cotransporter 2 inhibitors, have been shown to induce diabetic bone disorders via effects on bone and mineral metabolism (Palermo et al., 2015; Meier et al., 2016; Gilbert and Pratley, 2015). Recently, natural plants have attracted a great deal of interest as sources of alternative medicines for prevention and treatment of diabetes-related bone disorders.

Polygonum multiflorum Thunb. is a popular Chinese traditional medicinal herb, which is known as Heshouwu in China, Korea, and East Asia, and as Fo-ti in North America (Bounda and Feng, 2015). Heshouwu has a low toxicity and exhibits various pharmacological activities, such as anti-tumor (Horikawa et al., 1994; Choi et al., 2007), anti-inflammation (Cha and Jeon, 2009), nephroprotective (Guo et al., 2001), anti-atherosclerosis (Yang et al., 2005; Zhang et al., 2007), and anti-diabetes (Kang et al., 2005) activities. We previously showed that Heshouwu ethanol extract attenuates hyperglycemia and hyperinsulinemia in diet-induced obese mice (Choi et al., 2018). Hwang et al. (2016) and Kim et al. (2018b) reported that Heshouwu hot water extract and tetrahydroxystilbene glucoside (THSG), which is a bioactive compound of

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Heshouwu, prevent bone loss in estrogen deficient mice. Zhang et al. (2019) recently reported that THSG protects against diabetes-induced osteoporosis in mice with streptozotocin (STZ)-induced diabetes. However, it remains unknown if Heshouwu can regulate diabetes-induced bone loss *in vivo*, or the mechanism by which its effects occur. Therefore, we investigated the effects of Heshouwu water extract (HSW) on serum bone-related markers, BMD, bone quality parameters and bone metabolism-related gene levels in high-fat diet (HFD) and STZ-induced diabetic mice.

MATERIALS AND METHODS

Heshouwu (*Polygonum multiflorum* Thunb.) extract

Heshouwu (500 g) (Dong-Bu Herbal Makers, Suncheon, Korea) were extracted three times with water (each 5 L) by exhaustive maceration under sonication at 85°C for 3 h. The supernatant was filtered using Whatman number 2 filter paper (Whatman, Kent, England). The extracted fluid was concentrated with a rotary evaporator (EYELA, Tokyo, Japan) and freeze-dried to yield crude extract (25.2%).

Animal experiment

Male four-week-old C57BL/6N mice were purchased from Orient Bio Inc. (Seongnam, Korea). The animals were individually housed in polycarbonate cages and maintained in a temperature and humidity-controlled room (22±2 °C, 50±5% humidity) under a 12-h light-dark cycle. This study was approved by the Institutional Animal Care and Use Committee of Sunchon National University (approval number, SCNU IACUC-2016-11). All mice were acclimatized for 7 days, then fed HFD (40% calories from fat) for 4 weeks. After the HFD feeding period, mice were intraperitoneally injected with STZ (100 mg/kg body weight) dissolved in 0.1 M citrate buffer at pH 4.2 for 2 consecutive days. Only mice that exhibited a fasting blood glucose (FBG) level ≥250 mg/dL after 7 days were used in the study. The diabetic mice were randomly divided into two groups of eight mice each (control and HSW-treated group). In the present study, HSW was orally administered at 300 mg/kg body weight for 10 weeks, as described previously (Do et al., 2011).

During the experimental period, blood glucose levels and body weight were measured weekly and food consumption was measured daily. At the end of the experimental period, mice were anesthetized with ether and sacrificed after 12 h of fasting to collect blood and tissue samples for analysis. Blood samples were collected from the inferior vena cava and centrifuged (3,000 rpm for 15 min at 4°C) to separate out the serum. Tissues (liver, kidney, heart, and bone) were removed, rinsed with an ice-cold physiological saline solution, and weighed.

Micro-computed tomography (μCT) analysis

For μCT analysis of bone tissues (femurs and tibias), samples were isolated, cleaned of adherent soft tissues, and kept in 70% ethyl alcohol solution until scanning. To prevent moisture infiltrating, samples were wrapped with plastic wrap and placed into a holder. Morphometric parameters of bone samples were assessed using a μCT system (Skyscan 1272, Bruker, Kontich, Belgium). The scan setting were as follows: voltage of 70 kV, current of 142 μA, resolution step of 26.5 μm, and rotation step of 0.4°. 2D images were obtained for visualization and display. The image slices were reconstructed and analyzed using NReacon and CTAn (version 1.16.4.1, Bruker) software. BMD, bone volume/tissue volume ratio (BV/TV), bone surface/bone volume ratio (BS/BV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and structure model index (SMI) were then calculated. For quantification of trabecular BMD, the μCT was calibrated using two standard phantoms with a density of 0.25 and 0.75 g/cm^3. The distal femur and tibia metaphysic represented regions of interest for analysis.

Blood glucose, glycosylated hemoglobin (HbA_{1c}), serum insulin, and bone markers

The FBG concentration was monitored using a glucometer (G-doctor, AllMedicus Co., Ltd., Anyang, Korea). Venous blood was drawn from the tail vein every week after a six-hour fast, and the HbA_{1c} concentration in whole blood was measured using a NycoCard Reader II (Alere/Axis-Shield, Oslo, Norway). Serum insulin (Morinaga Institute of Biological Science, Yokohama, Japan), osteocalcin (OCN, Elabscience, Wuhan, China), bone-alkaline phosphatase (BAP, Elabscience), and tartrate-resistant acid phosphatase 5 (TRAP) (Cusabio Biotech, Wuhan, China) levels were determined using mouse enzyme-linked immunosorbent assay kits.

Quantitative polymerase chain reaction analysis

Total RNA isolation from bone tissue and mRNA expression (*NFATc1*: nuclear factor of activated T-cells, cytoplasmic 1, *RANKL*: receptor activator of nuclear factor kappa-B ligand, and *TRAP*) analysis were conducted as previously described (Ham et al., 2017). The sequences of the primers used are shown in Table 1. Gene expression was normalized against the *β-actin* gene.

Statistical analysis

All data are expressed as the mean±standard error (SE). The SPSS program was used to conduct all statistical analyses (SPSS Inc., Chicago, IL, USA). Data between groups were compared by Student's *t*-test. Correlations between

Table 1. Sequences of primers used for real-time PCR

Genes		Primer sequences (5′→3′)	Size (bp)
NFATc1	Forward	AGGACCCGGAGTTCGACTT	106
	Reverse	GTCGAGGTGACACTAGGGGA	
RANKL	Forward	CGAGGAAGGGAGAGAACGAT	92
	Reverse	AGGTACTTGCCGTAGTCTCG	
TRAP	Forward	AGGAAGAGCCTTCAAGTAAGTG	89
	Reverse	CCACCCATGAATCCATCTTCT	
β-Actin	Forward	GATCAGCAAGCAGGAGTACGA	91
	Reverse	GGTGTAAAACGCAGCTCAGTAAC	

NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; *RANKL*, receptor activator of nuclear factor kappa-B ligand; *TRAP*, tartrate-resistant acid phosphatase 5.

serum insulin levels and BMD were assessed by Pearson's correlation analysis. A $P<0.05$ was considered statistically significant for all analyses.

RESULTS AND DISCUSSION

Effects of HSW on blood glucose, HbA_{1c}, and insulin level

This study showed that HSW suppressed increases in FBG levels throughout the experimental period in HFD and STZ induced-diabetic mice; FBG levels were significantly lower (21%) in HSW mice compared with control mice at week 10 (Fig. 1A). THSG extracted from HSW has been shown to have hypoglycemic effects and to protect against nephropathy in mice and rats (Li et al., 2010; Chen et al., 2016; Zhang et al., 2012). HbA_{1c} levels reflect average blood glucose levels over the past 2~3 months (Khan and Weinstock, 2011); we therefore measured HbA_{1c} levels in the present study. Although not statistically significant, HSW-administered mice showed a decrease in HbA_{1c} levels approximately 10.4% (Fig. 1B). Since long-term hyperglycemia decreases body weight and insulin levels, we measured the effects of HSW on these parameters. The results demonstrated that HSW prevented body weight loss and increased serum insulin levels (Fig. 1C, D, and Table 2); however, HSW did not affect the weight of organs (liver, kidney and heart) or food intake (Table 2). Thus, these results indicate that HSW might exert anti-diabetic effects by increasing insulin levels.

Effects of HSW on microarchitecture and serum markers of bone conversion

Diabetic patients have a higher risk of bone fractures (Kanazawa, 2015). Because our coworkers previously indicated that HSW hot water extract and its bioactive component THSG prevent bone loss in an ovariectomy-in-

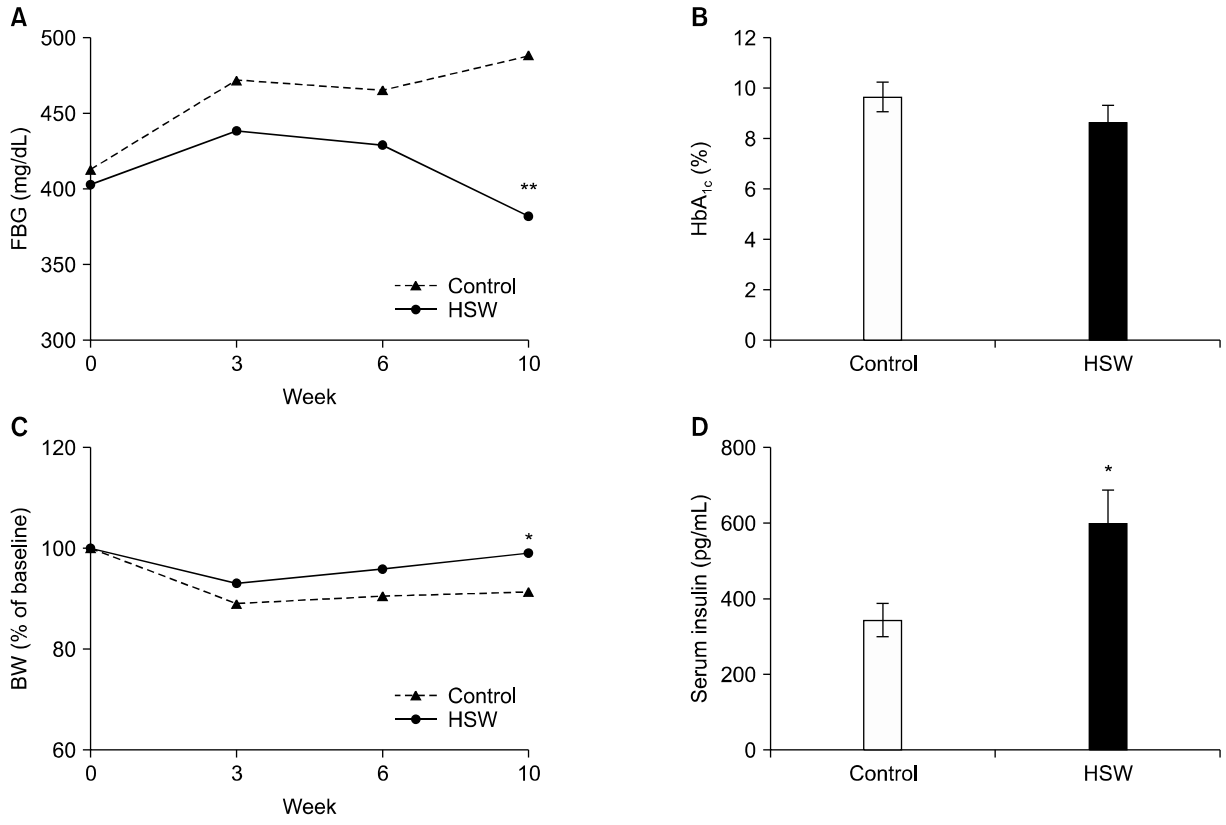

Fig. 1. Effects of Heshouwu water extract (HSW) on (A) fasting blood glucose (FBG), (B) HbA_{1c}, (C) change of body weights (BW), and (D) serum insulin levels in diabetic mice. Values are expressed as mean±SE. Statistical significance was assessed using the Student's *t*-test. $^{*}P<0.05$ and $^{**}P<0.01$ versus control.

Table 2. Effects of Heshouwu water extract (HSW) on body weight gain, food intake, and relative organ weights in diabetic mice

	Control	HSW
Body weight gain (g)	−2.35±0.59	−0.25±0.74*
Food intake (g/d)	3.80±0.08	3.54±0.14
Relative organ weights (mg/g)		
Liver	51.89±2.16	45.85±1.22
Kidney	16.52±0.59	15.10±1.90
Heart	4.92±0.11	4.66±0.23

Values are expressed as mean±SE.
Values are significantly different between groups according to Student's *t*-test.
*$P<0.05$ versus control.

duced osteoporosis mouse model (Hwang et al., 2016; Kim et al., 2018b), this study was conducted to determine if HSW extract may prevent bone loss caused by diabetes.

Osteoporosis is characterized by low bone mass and microstructural damage of the bone tissue (Li et al., 2019). Two dimensional μCT images of femoral and tibial trabecular and cortical bones revealed greater bone densities in the HSW group than the control group (Fig. 2A). BMD is the major quantitative indicator of osteoporosis (Li et al., 2019). In the present study, HSW significantly increased femoral BMD by 1.8-fold and tended to increase the tibial BMD by 1.3-fold compared to the control group (Fig. 2B). Interestingly, we found that serum insulin levels were positively associated with the BMD in the femur (r=0.690, $P<0.001$) and tibia (r=0.569, $P<0.05$), respectively (Fig. 2C). Compared with the control group, HSW significantly increased the BV/TV, Tb.Th, and Tb.N, and decreased the BS/BV and SMI (Fig. 3). In addition, HSW significantly decreased Tb.Sp in the femur (Fig. 3). Diabetes induces trabecular microarchitecture deterioration resulting in thinner and more rod-like trabeculae (higher SMI), which are more prone to bending deformation (Acevedo et al., 2018). Here, we found that HSW induced thicker, more plate-like trabeculae, and increased their numbers, indicating that bone strength was increased relative to the control group. These results suggest that HSW might increase bone mass and alleviate the trabecular microstructure in diabetic mice, while increasing insulin levels.

To confirm the anti-osteoporosis effects of HSW, we evaluated serum bone-related biochemical indexes. Compared with the control group, in the HSW group serum OCN and BAP were increased, and TRAP was decreased (Table 3). OCN plays a key role in mineralization and may be essential for osteoblast differentiation (Hienz et al., 2015). Kanazawa (2015) reported that OCN directly stimulates insulin secretion in the pancreas, which may be an important factor linking bone and glucose homeostasis. BAP is also an important marker of bone formation and bone turnover, and is used in the evaluation of skeletal status (Lim et al., 2016). In contrast, the increased TRAP in diabetic rats reflects a trend toward bone loss (Rivoira et al., 2018; Liu et al., 2018). Thus, HSW increases the bone formation markers OCN and BAP, and suppresses the bone resorption marker TRAP in diabetic mice.

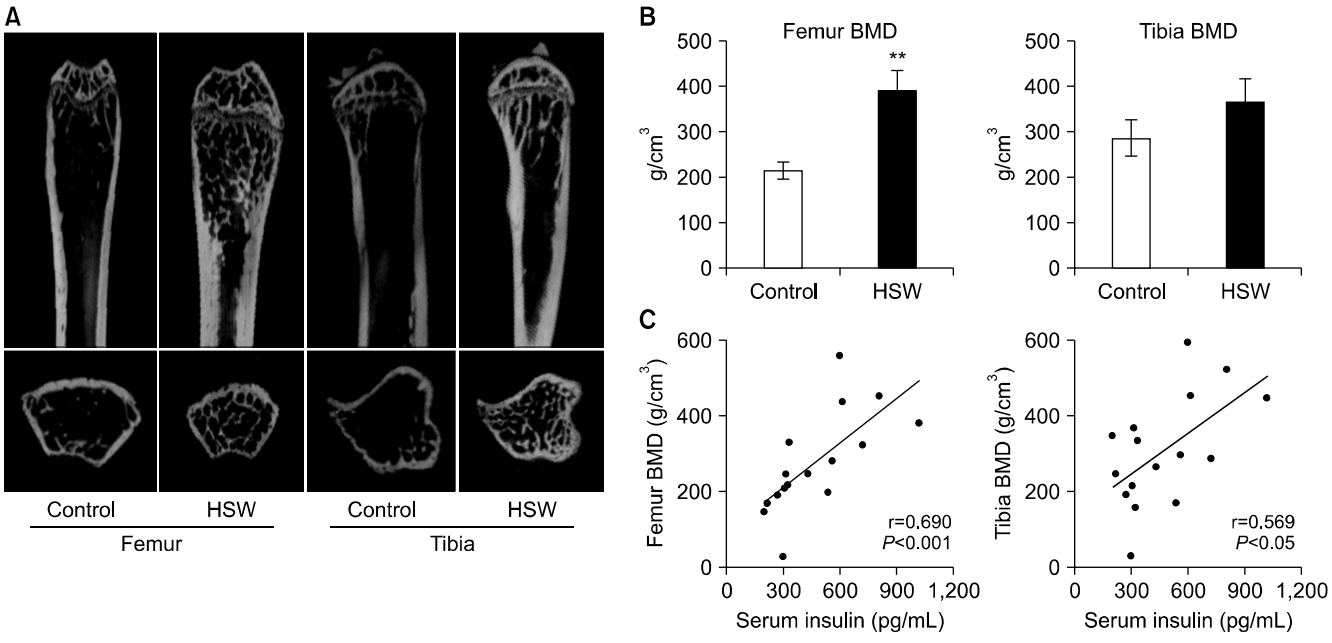

Fig. 2. Effects of Heshouwu water extract (HSW) on (A) bone micro-computed tomography image, (B) femur and tibia bone mineral density (BMD), and (C) correlation analysis of diabetic mice. Values are expressed as mean±SE. Statistical significance was assessed using the Student's *t*-test. **$P<0.01$ versus control.

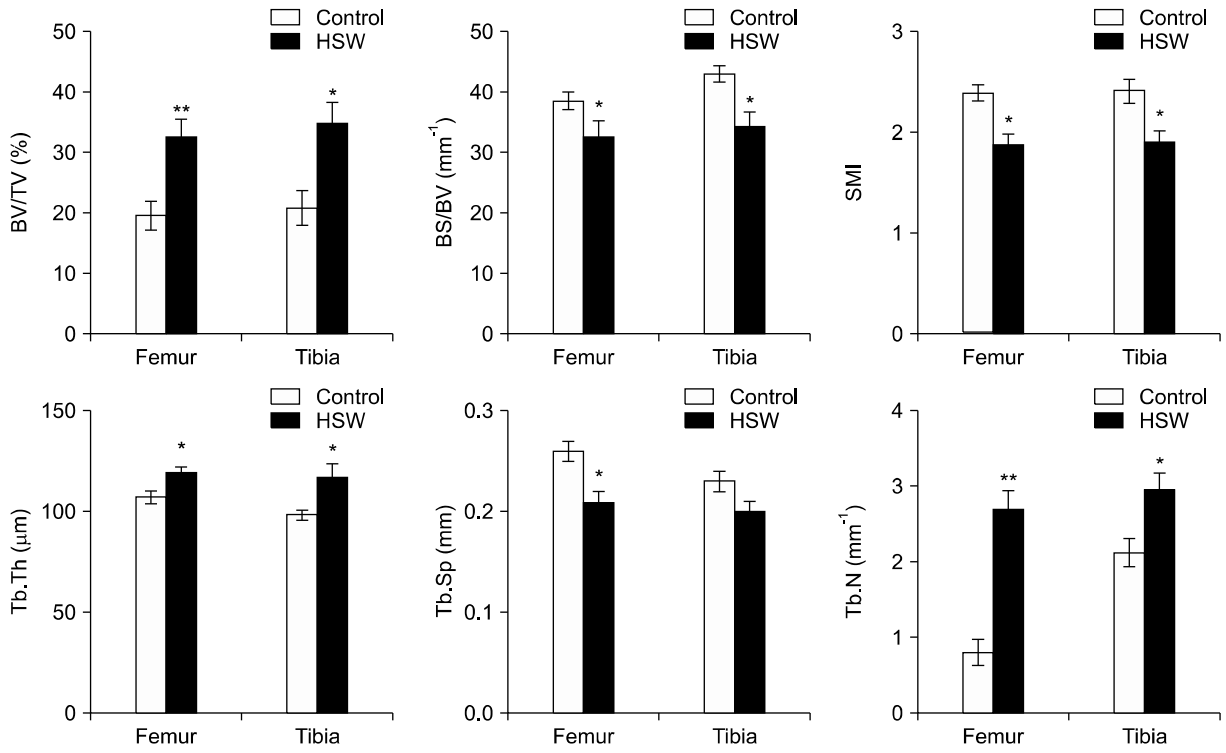

Fig. 3. Effects of Heshouwu water extract (HSW) on bone microarchitecture parameters in diabetic mice. Values are expressed as mean±SE. Statistical significance was assessed using the Student's *t*-test. **P*<0.05 and ***P*<0.01 versus control. BV/TV, bone volume/tissue volume ratio; BS/BV, bone surface/bone volume ratio; SMI, structure model index; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number.

Table 3. Effects of Heshouwu water extract (HSW) on serum bone biomarkers in diabetic mice (unit: ng/mL)

	Control	HSW
OCN	0.87±0.14	4.85±0.60**
BAP	0.90±0.27	2.27±0.39*
TRAP	61.27±2.33	49.79±1.14**

Values are expressed as mean±SE.
Values are significantly different between groups according to Student's *t*-test.
**P*<0.05 and ***P*<0.01 versus control.
OCN, osteocalcin; BAP, bone-alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase 5.

Effects of HSW on bone metabolism-related gene expression

We investigated the effect of HSW on bone formation and resorption-related gene expression. The results revealed that HSW significantly down-regulated osteoclastogenic genes, such as *NFATc1* and *TRAP*, in both the femur and tibia compared to the control group (Fig. 4). Bone remodeling is regulated by the balance between bone formation (osteoblasts) and bone resorption (osteoclasts) (Teitelbaum, 2000). Loss of this homeostasis leads to bone diseases such as osteoporosis, rheumatoid arthritis,

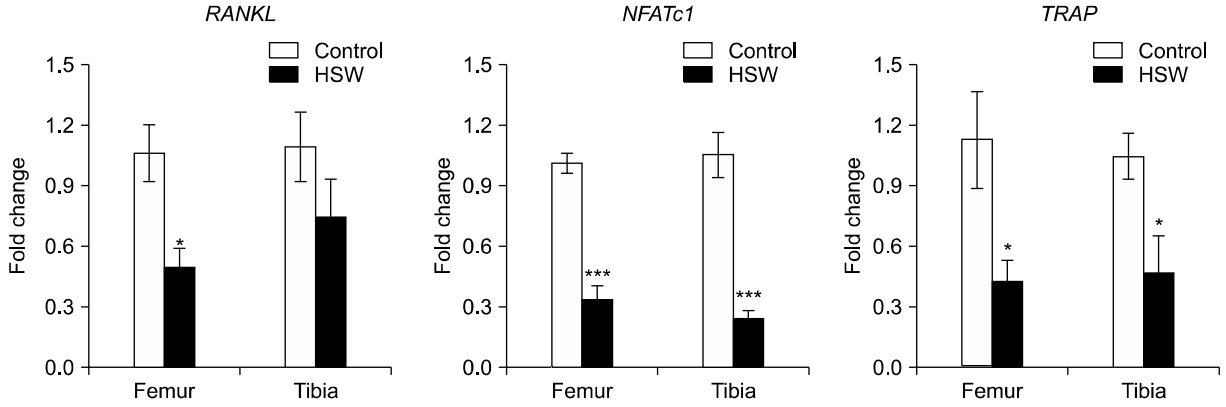

Fig. 4. Effects of Heshouwu water extract (HSW) on osteoclast-related gene expression of bone tissues in diabetic mice. Values are expressed as mean±SE. Statistical significance was assessed using the Student's *t*-test. **P*<0.05 and ****P*<0.001 versus control. *RANKL*, receptor activator of nuclear factor kappa-B ligand; *NFATc1*, nuclear factor of activated T-cells, cytoplasmic 1; *TRAP*, tartrate-resistant acid phosphatase 5.

and osteosarcoma. Osteoclasts are bone resorbing cells that are differentiated from the hematopoietic stem cells monocyte/macrophage lineage by RANKL (Kim et al., 2018a). In the present study, HSW decreased *RANKL* mRNA levels in the femur (52.8%) and tibia (31.2%) compared controls, although this decrease was only significant in the femur (Fig. 4). Binding of RANKL to its receptor on the surface of osteoclast precursor cells induces expression of important transcription factors for osteoclastogenesis, such as nuclear factor-κB, c-Fos, and NFATc1 (Kim and Kim, 2014). In particular, *NFATc1* is an indispensable factor for osteoclast differentiation *in vitro* and *in vivo*, which suggests that its regulation could be targeted by novel therapeutic strategies for bone diseases (Kim and Kim, 2014). *NFATc1* controls osteoclast-specific genes, such as *TRAP*, *cathepsin K*, and *calcitonin receptor* (Kim et al., 2018a). *NFATc1* expression in the femur ($r=0.546$, $P<0.05$) and tibia ($r=0.622$, $P<0.05$) were negatively correlated with serum BAP levels (data not shown). In the present study, HSW suppressed *NFATc1* expression in the femur and tibia and downstream *TRAP* expression, which corresponded to reduced serum TRAP levels relative to the control. Reducing osteoclast differentiation-associated pathways by HSW may therefore represent a potential method for therapeutic intervention to prevent diabetes-related bone loss. In contrast, a previous study showed that THSG increases osteoblastogenic genes such as *runt-related transcription factor 2* (*RUNX-2*), *OCN*, and *ALP* in STZ-induced diabetic mice (Zhang et al., 2019). Our data did not show significant changes in *RUNX-2*, *OCN*, and *ALP* between groups (data not shown). In conclusion, these results indicate that HSW might play a protective role in diabetes-induced osteoporosis, in part by down-regulating osteoclastogenesis.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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