EFFECT OF PUERARIN ON THE PROLIFERATION AND DIFFERENTIATION OF OSTEOBLASTS AND THE EXPRESSION OF TYPE I COLLAGEN mRNA IN A HIGH-GLUCOSE ENVIRONMENT

X.H. Wang*, X.W. Shi, X.X. Luo, D.H. Zhang

Gansu Provincial Hospital of Traditional Chinese Medicine, Department of Endocrinology, Lanzhou, China

Abstract

Objective. To investigate the effect of puerarin (Pue) on the proliferation and differentiation of osteoblasts and the expression of type I collagen(Coll I) mRNA in a high-glucose (HG) environment, and to provide evidence for the clinical treatment of diabetic osteoporosis(DOP).

Subjects and Methods. The proliferation of osteoblasts from three groups – the control group, the HS group, and the HG+Pue (10^{-8} - 10^{-6} M) group – was cultivated for 72 h and evaluated using the methyl thiazolyltetrazolium (MTT) assay.

Results. The MTT values and the ALP activities in all experimental groups were significantly lower than those in the control group, and the MTT values and the ALP activities in the HG+Pue group were significantly higher than those in the HS group. Coll I mRNA expression in all experimental groups was significantly lower than that in the control group, while that in the HG+Pue group was significantly higher than that in the HG group.

Conclusions. The proliferation and differentiation of osteoblasts and the expression of Coll I mRNA were inhibited by high glucose, but Pue can increase the proliferation and differentiation as well as the expression of Coll I mRNA in the osteoblasts, indicating that Pue could be therapeutically beneficial against DOP.

Keywords: type I collagen, differentiation, osteoblast, proliferation, puerarin.

INTRODUCTION

There are approximately 200 million people with osteoporosis globally. As a group of systemic skeletal diseases, osteoporosis (OP) can be divided into the following categories: primary osteoporosis, secondary osteoporosis, and unexplained idiopathic osteoporosis, among which diabetic osteoporosis (DOP) in secondary osteoporosis has become one of the chronic occult diseases that seriously endanger health and life quality nowadays. Within the secondary osteoporosis subcategory, endocrine osteoporosis is an important

type (1). Diabetic osteoporosis (DOP) is a systemic metabolic bone disease co-occurring in patients with diabetes mellitus (DM). This condition is characterized by a loss of bone mass and bone microstructure damage, as well as increased bone fragility and fracture occurrence. Insulin deficiency, insulin-like growth factor-1 deficiency, and the accumulation of advanced glycation end products (AGEs) in patients with DM can all promote the occurrence and development of DOP; however, the specific pathogenesis of DOP has not been fully elucidated (2). Hyperglycemia can also lead to the accumulation of AGEs in bone collagens, causing changes in its physical properties, blocking expression of osteoblast phenotypes, and increasing bone resorption, which will eventually lead to a reduction in bone strength (3). Epidemiological survey data have shown that, compared with general populations, the incidence of osteoporosis and the osteoporotic fracture risk are significantly higher in diabetes patients (4, 5), and there is no effective treatment program so far.

Previous studies have established that the plant *Pueraria lobata* contains various isoflavones including puerarin (Pue), daidzin, and daidzein, which exhibit significant hypoglycemic and lipid-lowering effects (6). Moreover, its effects on activating blood circulation and dissipating blood stasis have certain applications in the clinical treatment of DM (7, 8). In addition, studies have reported that Pue can reduce bone resorption, promote bone formation, and increase bone density, while its adverse effects in stimulating hyperplasia of the uterine tissue are less than those of estrogen (9). Studies have shown that Pue can compete with estrogen *in vitro* and exhibit estrogen/anti-estrogen-like effects, with relatively fewer side effects. Furthermore, Pue can improve bone metabolism, and may thereby help prevent or treat primary osteoporosis (10-12). Clinical observation has shown that Pue can significantly reduce the symptoms of DOP (13), but the exact mechanism is still unknown.

*Correspondence to: Xiaohui Wang MD, No. 418 Guazhou Road, Lanzhou, 730050, China, E-mail: xiaohuiwang6688@126.com

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Diabetic osteoporosis (DOP) refers to a systemic metabolic bone disease characterized by bone loss, bone structure damage, and an increase in bone fragility in patients with diabetes mellitus (DM). Indeed, DOP is one of the chronic complications of DM that occurs within the skeletal system (14).

Recent studies have found that the plant *Pueraria lobata* contains various isoflavones, including Pue, daidzin, and soybean flavonoids, which can reduce blood sugar and fat, expand cardiac and cerebral blood vessels, regulate vascular endothelial cell function, combat oxidation, reduce bone resorption, promote bone formation, and increase bone density (6, 9, 15). They can also competitively bind to estrogen receptors *in vitro* and thereby exhibit estrogen-like activities, which improve bone metabolism, and reduce the symptoms of DOP. The mechanism by which this occurs is still unknown (13,16).

MATERIALS AND METHODS

Culture of osteoblasts

The skulls of ten newly born SD rats (within 24 h; provided by the Animal Laboratory of Gansu University of Traditional Chinese Medicine, Certificate No. 2014315) were dissected and the periosteum and other soft tissues were rinsed with PBS and placed in a dish containing dulbeccos modified eagle medium (DMEM). The dissected tissue was then cut into ~1–3 mm³ pieces, and digested using trypsin at 37°C for ~2–3 min. After adding 15% fetal bovine serum (FBS)-containing DMEM to terminate the digestion, the cells were added to 4 mL of 0.1% type II collagenase for a 1 h digestion at 37°C. During this time the cells were repeatedly vortexed and mixed via pipetting. The digested product was then centrifuged at 1000 rpm at 4°C for 10 min, and the cell precipitate was collected and placed onto one dish containing 15% FBS-containing DMEM (also containing 100 U/mL penicillin and 100 µg/mL streptomycin). These cells were maintained under standard conditions (37°C, 5% CO₂, and saturated humidity) at a cell density of approximately 5 × 10⁴ cells/cm². The primary cells obtained were kept in culture for 24 h, after which the medium was changed. Following this initial change, the media were changed once every 2–3 days. An inverted phase contrast microscope was used the next day to observe cell appearance and growing status. When the cells fused and formed one layer, they were used for the subculture. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the

National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Gansu Provincial Hospital of Traditional Chinese Medicine.

Morphologic observation

After being cultured for 48 h, the cells were fixed with 4% paraformaldehyde, rinsed using phosphate buffered saline (PBS) three times, and subjected to HE staining for the microscopic observation of the osteoblast morphology. Meanwhile, 100 µL 5-Bromo-4-Chloro-3-Indolyl Phosphate/nitroblue tetrazolium (BCIP/NBT) staining solution (Alkaline Phosphatase Color Development Kit, for developing the intracellular or intra-tissue endogenic alkaline phosphatase) was prepared and added into each well of a 48 well plate. This was followed by a ~5–30 min incubation of this solution in the dark at 25°C, until the color was developed to the desired depth. Subsequently, rinsing in distilled water halted color development. The number of osteoblast-like cells per well was counted under an inverted microscope (OLYMPUS, Tokyo, Japan), and the number of cells with positive alkaline phosphatase (ALP) staining was also counted.

Grouping and administration

When cell morphology became stable, the osteoblasts in the logarithmic growth phase were added into each well of a 96 well plate, and randomly divided into four groups: the normal group (N), 2) the high-glucose (HG) group (HS, glucose concentration 22 mM), 3) the Pue group (P, National Institutes for Food and Drug Control, different concentrations: 1 × 10⁻⁸M, 1 × 10⁻⁷ M, and 1 × 10⁻⁶ M), and 4) the HS+Pue group (with the same Pue concentrations as shown above). Each group had eight wells, and the cells were cultured for 48 h. The culture medium and the drugs were renewed every other day.

Methyl thiazolyltetrazolium (MTT) assay

The 1st generation cells were digested using 0.25% trypsin (Huamei Co., Shanghai China) and 0.02% EDTA. The cells were subsequently counted and seeded into the wells of a 96-well plate (at 2 × 10³ cells/well) containing MEM (GIBCO, USA) and 10% FBS (SJQ Co., Hangzhou China). The cells were then incubated at 37°C in 5% CO₂, and observed 24 h later when the cells were adherent and growing stably. After discarding the liquid supernatant and administering Pue for 72 h, cell proliferation was evaluated using the MTT assay, and the results were expressed by the OD

values measured using a microplate reader (Bio-Rad Model 550, Tokyo, Japan) at 490 nm.

The p-Nitrophenyl phosphate (PNPP) method was used to detect osteophosphorase (ALP) activity

The 1st generation cells were digested with 0.25% trypsin and 0.02% EDTA. The cells were subsequently counted and seeded into the wells of a 96-well plate (at 2×10^3 cells/well) in MEM containing 10% FBS. These cells were then incubated at 37°C in 5% CO₂, and observed 24 h later when the cells were adherent and growing stably. After discarding the liquid supernatant and administering drugs for 72 h, the cells' ALP activity was analyzed using the PNPP (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The results were expressed as the OD values measured using a microplate reader (Bio-Rad Model 550, Tokyo, Japan) at 405 nm.

RT-PCR measurement of type I collagen mRNA level in osteoblasts

The 1st generation cells were digested using 0.25% trypsin and 0.02% EDTA. The cells were then counted and seeded into 100 mL culture flasks (at 5×10^4 cells/well) in MEM containing 10% FBS. These cells were then incubated at 37°C in 5% CO₂ and observed after 24 h. Cells were maintained in culture for 10 more days until they were adherent and growing stably. After discarding the liquid supernatant, Trizol reagent (TaKaRa, Dalian, China) was added to each collected sample, along with 0.2 mL CHCl₃. The samples were then agitated vigorously for 15 sec, left to stand for 5 min at room temperature, and centrifuged for 15 min at $12,000 \times g$ at 4°C so as to extract the total RNA. The supernatant was then transferred to another centrifugation tube and an equal volume of isopropanol was added to the samples. These solutions were left to stand for 10 min at room temperature, after which they were centrifuged at $12,000 \times g$ for 10 min at 4°C. The RNA precipitated at the bottom of the tube, and its purity was subsequently checked using ultraviolet spectroscopy. One microliter of the above RNA was then diluted with 49 µL of RNase-free water for future use. RNase-free water was added into a micro-quartz cuvette (the cuvette was pre-rinsed with RNase-free water), and then used to determine the absorbance of the RNA solution at 260 and 280 nm. Simultaneously, the R value was also calculated as $R = OD_{260}/OD_{280}$, which can reflect the contamination level of the sample by substances such as protein. A sample of sufficient purity will have an R value between ~1.7 and 2.2;

when $R < 1.7$, there is significant protein contamination, and when $R > 2.2$, the RNA has been hydrolyzed into mononucleotides. The RNA concentration was determined using the following equation, $[RNA] = OD_{260} \times \text{dilution factor} \times 0.04 \mu\text{g}/\mu\text{L}$.

cDNA was produced using a program that lasted 15 min, and the reverse transcriptase was inactivated by exposing the sample to a temperature of 85°C for 5 s, followed by 1 h inactivation at 4°C. The relative expression of Coll I mRNA was detected by the RT-PCR (TaKaRa, Dalian, China). The primer sequences for Coll I mRNA: upstream primer 5'-TACAGCACGCTTGTGGATG-3', downstream primer 5'-TTGGGATGGAGGGAGTTTA-3', with an expected fragment size of 320 bp. The primer sequences for the internal reference GAPDH: upstream primer 5'-TGAACGGGAAGCTCACTGG-3', downstream primer 5'-TCCACCACCCTGTTGCTGTA-3', with an expected fragment size of 200 bp. The PCR conditions were a pre-denaturation step at 95°C for 10s, denaturation at 95°C for 5 s, and 72°C for 30 s, for a total of 40 cycles. The dissolution curves were prepared at 95°C for 60 s, 57.6°C for 30 s, and 95°C for 30s, for a total of one cycle. The products were separated via gel electrophoresis using a 1.7% agarose gel, and then analyzed using the YLN-2000 gel imaging analysis system (TechComp Co., Shanghai, China). GAPDH was used as the internal reference, and the relative content of the target gene was represented by the absorbance ratio of the target gene to the internal reference.

Expression of osteoblast type I collagen RT-PCR results showed 2 amplified bands at 320bp and 200bp, which were the same as Coll I and GAPDH. Puerarin increased Coll I mRNA expression in bone cells at different concentrations ($P < 0.05$, $P < 0.01$) and was significantly different with HG+Pue group ($P < 0.01$) (Figs 1, 2).

Statistical analysis

SPSS15.0 was used for statistical analysis and data processing. The data were expressed as mean $\pm$ standard deviation ($\pm s$), and the comparison among multiple groups used the single-factor analysis, with $P < 0.05$ considered as statistical significance.

RESULTS

Morphological observations and the identification of osteoblasts

Figure 3A shows that the cultured rat osteoblasts exhibit triangular, polygonal, or long

Figure 1. Gel electrophoresis imaging of Coll I mRNA in bone cells. Note: 1 HS, 2 Pue (10^{-8} mol/L), 3 HS+Pue (10^{-8} mol/L), 4 Pue (10^{-7} mol/L), 5 HS+Pue (10^{-7} mol/L), 6 Pue (10^{-6} mol/L), 7 HS+Pue (10^{-6} mol/L).

Figure 2. Gel electrophoresis imaging of Coll I mRNA in bone cells. Note: 1 HS, 2 control group, 3 HS+Pue (10^{-8} mol/L), 4 Pue (10^{-7} mol/L), 5 HS+Pue (10^{-7} mol/L).

Figure 3. Identification of osteoblasts. A: Morphologies of osteoblasts (40 $\times$); B: ALP chemical staining (40 $\times$).

Figure 4. Gel electrophoresis of Coll I mRNA in each group. Note: 1. group HS; 2. Pue (10^{-8} mol/L); 3. HS+Pue (10^{-8} mol/L); 4. Pue (10^{-7} mol/L); 5. HS+Pue (10^{-7} mol/L); 6. Pue (10^{-6} mol/L); 7. HS+Pue (10^{-6} mol/L).

fusiform shapes on the third day of culture, with rich cytoplasm, round nuclei, and clear outlines under the inverted microscope. The ALP qualitative staining (Fig. 3B) reveals that the cytoplasm of the osteoblasts is positive (stained blue).

Comparison of proliferation

Compared with group N, the OD values, and hence the amount of proliferation, in several of the Pue groups significantly increased (Table 1, $P < 0.01$), while those in group HS and HS+Pue (at various

concentrations) significantly decreased (Table 1, $P < 0.05$, $P < 0.01$, respectively). Compared with group HS, cell proliferation in HS+Pue group (at various concentrations) significantly increased, and the differences were statistically significant (Table 1).

Comparison of ALP activity

Compared with group N, the ALP activities in various Pue groups significantly increased (Table 2), while those in group HS and HS+Pue (at various concentrations) significantly decreased (Table 2). Moreover, the ALP activities in group HS were significantly higher than those in the HS+Pue group (at various concentrations; Table 2).

Comparison of Coll I mRNA expression

The RT-PCR results showed that amplified bands at the molecular weights of approximately 320 bp and 200 bp can be observed, corresponding to the expected amplified fragments of Coll I and GAPDH, respectively. The expression of Coll I mRNA in various Pue groups increased significantly as compared to group (Fig. 4, Table 3). The expression of Coll I mRNA in the

Table 1. Comparison of proliferation of osteoblasts among the groups ($\bar{x} \pm s$, n=8)

Group	Conc. (mol/L)	OD
N		0.561±0.063
HG		0.245±0.018 ^{##}
P	10 ⁻⁸	0.745±0.085 ^{####}
	10 ⁻⁷	0.737±0.030 ^{####}
	10 ⁻⁶	0.735±0.016 ^{####}
	10 ⁻⁸	0.442±0.025 ^{***}
HG+P	10 ⁻⁷	0.436±0.036 ^{**}
	10 ⁻⁶	0.429±0.045 ^{**}

Note: Compared with group N, #P<0.05, ##P<0.01; compared with group HG; *P<0.05, **P<0.01.

Table 2. Comparison of ALP activity of osteoblasts among the groups ($\bar{x} \pm s$, n=8)

Group	Conc. (mol/L)	OD
N		1.091±0.041
HG		0.54±0.023 ^{##}
P	10 ⁻⁸	1.378±0.026 ^{####}
	10 ⁻⁷	1.308±0.026 ^{####}
	10 ⁻⁶	1.268±0.030 ^{####}
	10 ⁻⁸	0.665±0.023 ^{***}
HG+P	10 ⁻⁷	0.609±0.014 ^{**}
	10 ⁻⁶	0.621±0.022 ^{**}

Note: Compared with group N, #P<0.05, ##P<0.01; compared with group HG; *P<0.05, **P<0.01.

Table 3. Comparison of expression of Coll I mRNA among different groups

Group	Conc. (mol/L)	OD
N		0.801±0.043
HG		0.453±0.032 ^{##}
P	10 ⁻⁸	1.154±0.024 ^{####}
	10 ⁻⁷	0.958±0.036 ^{####}
	10 ⁻⁶	0.864±0.010 ^{####}
	10 ⁻⁸	0.625±0.023 ^{***}
HG+P	10 ⁻⁷	0.529±0.024 ^{**}
	10 ⁻⁶	0.516±0.022 ^{**}

Note: Compared with group N, #P<0.05, ##P<0.01; compared with group HG; *P<0.05, **P<0.01.

HG group was significantly lower, when compared to the HG+Pue group (at various concentrations) (Fig. 4, Table 3, P<0.01).

DISCUSSION

With an aging population, as well as the changes of diet and lifestyle, the morbidity and detection rate of DM are increasing rapidly. It is expected that the global number of DM cases will exceed 300 million by 2025, with 38 million being present in China (17). As a metabolic disease, DM affects bone metabolism and remodeling, resulting in bone loss and osteoporosis. As a chronic complication of DM, DOP is a systemic bone

disease characterized by bone loss, bone microstructure degradation, bone fragility, and increased incidence of fracture. Studies have found that the incidence rate of osteoporosis in patients with DM is approximately 50% (18), and DOP continues to increase with increasing age and islet failure. Given its increased prevalence and the high incidence of morbidity and mortality, DOP is being paid more attention.

Studies have shown that estrogen affects osteoblast proliferation, differentiation, and apoptosis (19). Estrogen directly affects bone formation mainly through three mechanisms, namely inhibiting apoptosis, inhibiting the oxidative stress response, and reducing the NF- κ B activity (20). Pue is one of the main components of the Chinese medicinal plant, *P. lobata*, and it has a chemical structure similar to that of estrogen. In recent years, studies have confirmed that Pue can be used to prevent and treat postmenopausal osteoporosis, showing an estrogen-like effect while being less toxic (19, 20). Its ability to activate blood circulation and dissipate blood stasis has certain applications in the clinical treatment of DM (21-23). Osteoporosis is associated with degenerative changes. Various factors inhibit the osteoblast-mediated bone formation and promote the osteoclast-mediated bone resorption, thus resulting in greater bone resorption than bone formation and leading to a decrease in bone mineral density (BMD). The purpose of this study was to observe the effect of Pue on the proliferation and differentiation of the osteoblasts, as well as to assess its effects on the expression of Coll I mRNA in a high glucose environment, so as to understand the therapeutic effect of puerarin on the inhibition of osteoblast-mediated bone formation in high glucose environment.

The osteoblasts originate from the pluripotent bone marrow stromal cells, are involved in the three stages of bone formation (namely the cleavage and proliferation, differentiation and maturation, and stromal and calcification), and play important roles in bone remodelling and maintaining normal bone transformation. Cell proliferation is the most important basic indicator to reflect the cell growth, metabolism, and activity. MTT can indirectly reflect the number of active cells. In this study, the osteoblast activity was detected by MTT, and the results show that the proliferation of osteoblasts in high glucose environment decreased significantly, and the OD value of each concentration group was increased after puerarin intervention, indicating that high glucose environment inhibits the proliferation of osteoblasts, and puerarin can reduce the inhibitory effect of high glucose on the osteoblasts.

ALP is an extracellular enzyme of osteoblasts. The main function of ALP is to hydrolyze the phospholipids during osteogenesis. The released phosphate and calcium then deposit on the collagen skeleton and bone mineralization occurs. Meanwhile, the hydrolyzed pyrophosphate can relieve its inhibition toward the formation of bone salt, thus being conducive to the formation of bones. The activity of ALP is an important indicator for reflecting the differentiation of osteoblasts, the higher the activity, the more obvious the differentiation of osteoblasts (24). In this study, the internationally recognized PNPP method was used to detect the ALP activity in the osteoblasts, and the study finds that the OD value of the high glucose group decreased more significantly than the normal group, while puerarin intervention can increase the OD value of each experimental group, and the differences are statistically significant, indicating that high glucose environment can decrease the activity of ALP and also decrease the differentiation of osteoblasts, but puerarin can alleviate the effect of high glucose on the activity of ALP in the osteoblasts.

The bone matrix is the basis of bone tissue, and its chemical compositions include organic and inorganic components. Organic constituents are mainly type I collagen (Coll I), which makes the bones tough and elastic and being able to absorb external energy; at the same time, Coll-formed three-dimensional network structure is the premise of mineralization, so the structure and composition of collagen fibers limit the size and deposition direction of Ca-P crystal. The inorganic components are minerals, which provide the stiffness of bones. Coll I is secreted by the osteoblasts, and its content *in vitro* is consistent with the ability of bone formation by the osteoblasts. In this study, RT-PCR was used to detect the mRNA expression of type I collagen in the osteoblasts. The results show that the Coll I mRNA expression is decreased in a high glucose environment while it is increased in each puerarin intervention group, and the differences are statistically significant, indicating that puerarin can improve the low mRNA expression of Coll I in high glucose environment.

In conclusion, this study shows that the balance between osteoclastic/osteogenic activity is disrupted in high glucose environments, thus leading to DOP, but the active ingredients, mainly the isoflavones, in *P. lobata* can restore the above balance and maintain normal bone mass and bone quality. However, if Pue is to be clinically used for the prevention and treatment of DOP, further in-depth studies must be carried out to determine proper dosages, side effects, and mechanisms. The

continuous deepening of modern medical science can provide a theoretical and experimental basis for the use of phytoestrogens, such as those present in *P. lobata*, as a new treatment against DOP with more selectivity and safety.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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