Original Article

Effects of Anastrozole Combined with Shuganjiangu Decoction on Osteoblast-like Cell Proliferation, Differentiation and OPG/RANKL mRNA Expression

Yan Ren, Shu-yan Han, Ping-ping Li*

Key Laboratory of Carcinogenesis and Traditional Research (Ministry of Education), Department of Integrated Traditional Chinese and Western Medicine, Peking University Cancer Hospital & Institute, Beijing 100142, China

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ABSTRACT

Objective: To investigate the effects of anastrozole combined with Shuganjiangu decoction on osteoblast-like cells.

Methods: Human osteoblast-like cells MG-63 were cultured and divided into four groups: control, anastrozole, Shuganjiangu decoction (SGJGD), and anastrozole combined with SGJGD. Cell proliferation was investigated by MTT assay. Alkaline phosphatase (ALP) and osteocalcin, the indicators of cell differentiation, were evaluated by p-nitrophenyl-phosphate method and radioimmunoassay, respectively. Gene expressions of ALP, osteocalcin, osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) were examined by real-time PCR.

Results: As evidenced by MTT assay, cell proliferation of MG-63 was inhibited by anastrozole, but stimulated with treatment of SGJGD alone and combined with anastrozole (P<0.01). Compared with control group, ALP activity was increased by the treatment of SGJGD alone and combined with anastrozole (P<0.01). Also, osteocalcin secretion was enhanced with the treatment of SGJGD single and combination with anastrozole (P<0.05). In the real-time PCR assay, gene expressions of ALP and osteocalcin were significantly increased (P<0.01 for ALP, P<0.05 for osteocalcin) by the treatment of SGJGD and anastrozole combined with SGJGD, but the expression of RANKL was decreased (P<0.05). Moreover, anastrozole combined with SGJGD upregulated gene expression of OPG (P<0.01).

Conclusion: SGJGD may alleviate the injury effects of anastrozole on MG-63 cells through adjusting bone formation and resorption indicators.

Key words: Shuganjiangu decoction; Osteoblast-like cell; Alkaline phosphatase; Osteocalcin; Osteoprotegerin; Receptor activator of nuclear factor kappa B ligand (RANKL)

INTRODUCTION

The survival from estrogen-dependent breast cancer has increased over the last decades due to improved endocrine therapy[1]. One of the major advances was the introduction of aromatase inhibitors (AIs), especially the third-generation nonsteroidal (anastrozole, letrozole) and steroidal (exemestane). AIs could suppress circulating estradiol to almost undetectable level, thus reduce the risk of disease recurrence in patients with estrogen receptor-positive (ER+) breast cancer[2-5].

However, estrogen played an important role in the maintenance of bone health. The marked reduction in circulating estrogen levels had adverse effects on bone physiology. The clinical studies confirmed that AIs therapy was associated with increased rate of bone loss and fracture events[6,7].

Shuganjiangu decoction (SGJGD), a traditional Chinese herbal formula, has been used to prevent AIs treatment-induced bone loss in clinics. SGJGD was originated from Shuganliangxue decoction with little modification. Our research showed Shuganliangxue decoction could inhibit breast cancer cells proliferation and down-regulate steroid sulfatase expression[8,9]. In the previous study, we found SGJGD also inhibited MCF-7 cell proliferation and was without estrogenic activity *in vitro*. It is interesting to find that SGJGD promoted the proliferation of MG-63 cells, a commonly used osteoblast-like cell line, while Shuganliangxue decoction had no influence on it. However, the underlying mechanism of SGJGD for increasing osteoblast cell proliferation was unknown, and the combined effect of

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*Corresponding author.
E-mail: lppma123@yahoo.com.cn

SGJGD with anastrozole on osteoblast cells also deserved further research. Thus, the objective of present research was to investigate the effects of anastrozole combined with SGJGD on MG-63 cells and illustrate the action mechanisms.

MATERIALS AND METHODS

Cell Culture

Human osteoblast-like cell line MG-63 was purchased from Cell Resource Center (CRC/PUMC, China). MG-63 cells were routinely cultured in Dulbecco modified Eagle's medium (DMEM, TianRunShanda, Beijing, China), supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and maintained at 37°C in 5% CO_2 humidified atmosphere.

Preparation of SGJGD

SGJGD consisted of *Radix Bupleuri* 10 g, *Radix curcumae* 10 g, *Lithospermum Erythrorhizon Sieb.et Zucc* 15 g, *Radix Paeoniae Alba* 15 g, *Cortex moutan* 10 g, *Rhizoma drynariae* 15 g, *Concha ostreae* 10 g, and *Fructus schisandrae chinensis* 10 g, which were purchased from Bai-Ta-Si drugstore in Beijing. All herbs were soaked in distilled water for 30 min and extracted twice by boiling them in water for 1 h. Then the extract was centrifuged at 6,000 r/min for 20 min and concentrated to 1.2 g/ml by water bath. The pH was adjusted to 7.0–7.1. The concentrated extract was filtered through a 0.22 μm pore-size membrane (Millipore, USA)[8].

Cell Proliferation Assay

MG-63 cells were seeded in a 96-well plate at a density of 4,000 cells per well, cultured for 24 h, and then treated with SGJGD and anastrozole alone or in combination for 24 h or 48 h, respectively. At the end of the treatment, the cells were washed twice with PBS, and the culture medium containing 10% 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) was added. After incubation with MTT at 37°C for 4 h, 100 μl dimethyl sulfoxide (DMSO) was added to each well, followed by gently shaking for 10 min to achieve complete dissolution. The absorbance was determined at 570 nm[10]. The half maximal inhibitory concentration (IC50) value was defined as the concentration needed for 50% reduction in the absorbance, as calculated based on the non-linear regression fit method by Graphpad Prism 4.0 software (San Diego, CA, USA).

ALP Activity Assay

MG-63 cells were cultured in a 6-well culture plate at a density of $6×10^4$ cells per well for 24 h. After treated with SGJGD and anastrozole alone or in combination for 48 h, the cells were lysed with 0.05% Triton X-100 (Amresco, USA). ALP activity was determined by converting p-nitrophenyl phosphate to p-nitrophenol using a commercial kit (Nanjing Jiancheng, China)[11]. The sample (20 μl) was mixed with 100 μl substrate, and the mixture was incubated at 37°C for 15 min. After the incubation, 80 μl reaction-stop solution was added and the absorbance was determined at 405 nm. The total protein concentration of samples was evaluated with a bicinchoninic acid (BCA) protein assay kit (Thermo, USA), and the absorbance in each sample was normalized based on the protein content.

Radioimmunoassay for Osteocalcin

MG-63 cells were cultured at a density of $6×10^4$ cells per well for 24 h and treated with SGJGD and anastrozole alone or in combination for 48 h. The conditioned medium was collected and freeze-dried, and the osteocalcin concentration was measured by a radioimmunoassay (RIA) kit purchased from RIA institute of 301 Hospital (Beijing, China). The sample was dissolved in 100 μl PBS, and mixed with 100 μl I^{125}-osteocalcin and 100 μl osteocalcin antibody. After incubated at 37°C for 24 h, 500 μl separating agent was added into the mixture for 20 min. The final mixture was centrifuged at 3,500 r/min for 15 min, and the precipitate was counted to calculate osteocalcin content.

Real-time PCR

Total RNA was extracted from the cells by Trizol reagent (Transgen, China), and the first-strand cDNA was generated by TransScript first-strand cDNA synthesis supermix (Transgen) according to the manufacturer's instruction. Primers designed for real-time PCR were showed in Table 1. The real-time PCR assay was carried out by trans-start green qPCR supermix (Transgen) and performed in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA). The relative expression of target gene mRNA was quantified by measuring the cycle threshold (CT) and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The fold change was determined by the $2^{-\Delta\Delta CT}$ method[12].

Statistical Analysis

Results were expressed as $\bar{x}±s$. Differences among cell viability, ALP activity and mRNA, osteocalcin secretion and mRNA, and osteoprotegerin (OPG)/ receptor activator of nuclear factor kappa β ligand (RANKL) mRNA in MG-63 cells were analyzed by one-way analysis of variance (ANOVA) using statistical analysis software SAS 9.1 (SAS Institute, Raleigh, NC, USA). For this research, the statistical significance level was set at $P<0.05$.

RESULTS

Effects of Anastrozole and SGJGD Alone or in Combination

on Cell Viability of MG-63

As shown in Figure 1, MG-63 cell viability was not affected by the treatment of 10 nmol/L to 100 μmol/L anastrozole for 24 h. Cell toxicity appeared at 100 μmol anastrozole for 48 h treatment and the cell viability was decreased to 78%. SGJGD (0.046875–12 mg/ml) slightly increased the viability of MG-63 cells at 24 h, but the cell viability was increased dose-dependently by SGJGD treatment for 48 h, and the concentration between 0.75 mg/ml and 12 mg/ml showed significance when compared with the control group (Figure 2).

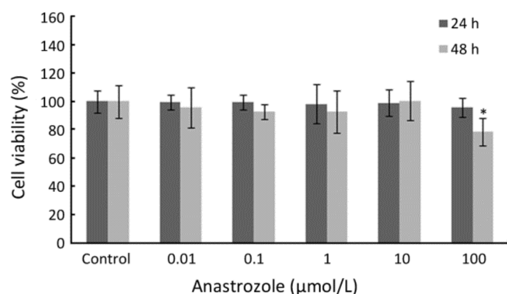

Figure 1. Effects of anastrozole on viability of MG-63 cells by MTT assays. Cells were treated with various concentrations of anastrozole for 24 h and 48 h. Data were represented as $\bar{x}\pm s$ of at least three independent experiments and calculated as percentage of control values. $^{*}P$<0.05, compared with 0 μmol/L anastrozole.

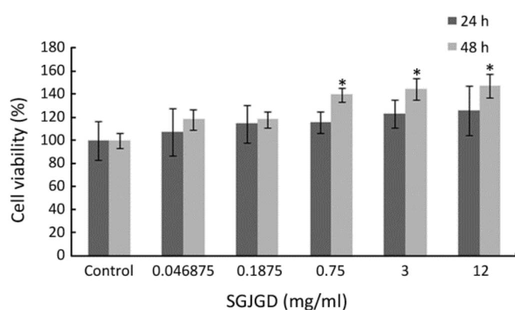

Figure 2. Effects of SGJGD on viability of MG-63 cells by MTT assays. Cells were treated with various concentrations of SGJGD for 24 h and 48 h. Data were represented as $\bar{x}\pm s$ of at least three independent experiments and calculated as percentage of control values. $^{*}P$<0.05, $^{**}P$<0.01, compared with 0 mg/ml SGJGD.

Based on these experiments, we chose 100 μmol/L anastrozole and 12 mg/ml SGJGD to do the following experiments. Figure 3 showed anastrozole caused significant decrease of MG-63 cell viability after 48 h treatment (*P*<0.05 vs. control), while SGJGD significantly increased the cell viability (*P*<0.01). The combination of anastrozole and SGJGD showed a similar effect on MG-63 cells and also increased cell growth (*P*<0.01).

Effects of Anastrozole and SGJGD Alone or in Combination on ALP Activity and mRNA Expression in MG-63 Cells

As shown in Figure 4, anastrozole treatment did not influence the ALP activity in MG-63 cells. However, the treatment of SGJGD obviously stimulated the ALP activity (*P*<0.01). When MG-63 cells were treated with the combination of anastrozole and SGJGD, ALP activity was increased when compared with the treatment with anastrozole alone (*P*<0.01).

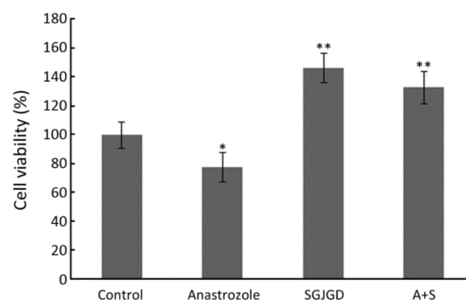

Figure 3. Effects of anastrozole, SGJGD alone and in combination on the viability of MG-63 cells. Cells were treated with control, anastrozole 100 μmol/L, SGJGD 12 mg/ml and anastrozole 100 μmol/L+SGJGD 12 (A+S) for 48 h, and MTT assays were performed. Data were represented as $\bar{x}\pm s$ of at least three independent experiments and calculated as percentage of control values. $^{*}P$<0.05, $^{**}P$<0.01, compared with control group.

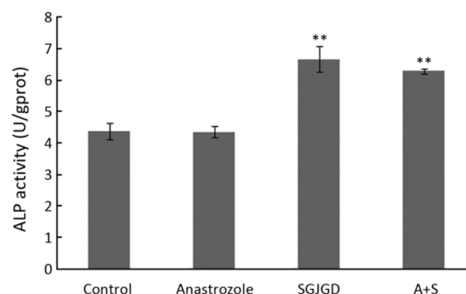

Figure 4. Effects of anastrozole, SGJGD alone and in combination on ALP activity of MG-63. Cells were treated with control, anastrozole 100 μmol/L, SGJGD 12 mg/ml and anastrozole 100 μmol/L + SGJGD 12 mg/ml (A+S) for 48 h, and ALP activity was determined. Results were normalized by the total protein content and expressed as percentage of control values. $^{**}P$<0.01, compared with control group.

Real-time PCR (Figure 5) showed that the mRNA expression of ALP was increased by the treatment of SGJGD alone or combined with anastrozole, compared with control group (*P*<0.01). No obvious change was observed in anastrozole group.

Effects of Anastozole and SGJGD Alone or in Combination on Osteocalcin Secretion and mRNA Expression in MG-63 Cells

The effects of anastrozole and SGJGD alone or in

combination on osteocalcin secretion in MG-63 cells were shown in Figure 6. When compared with control, the osteocalcin secretion was slightly decreased by anastrozole in MG-63 cells, but was significantly increased by SGJGD alone or combined with anastrozole (P<0.05). Osteocalcin secretion was significantly increased by the addition of SGJGD, compared with anastrozole (P<0.05).

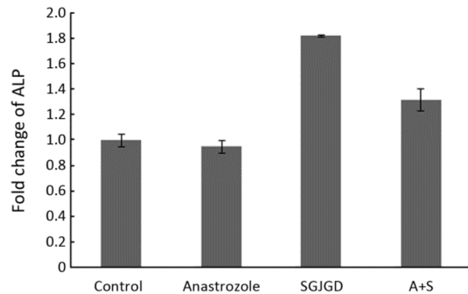

Figure 5. Expression of ALP mRNA in MG-63 exposed for 48 h to control, anastrozole 100 μmol/L, SGJGD 12 mg/ml or a combination of anastrozole 100 μmol/L + SGJGD 12 mg/ml (A+S). The fold change of ALP expression is calculated by the $2^{-\Delta\Delta CT}$ method. $^{**}P$<0.01, compared with control group.

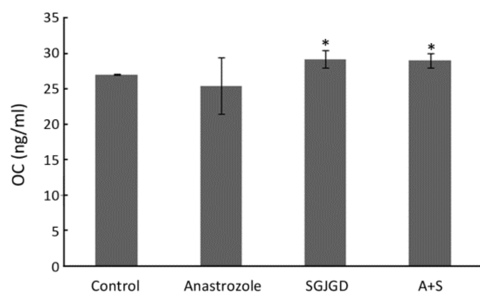

Figure 6. Effects of anastrozole, SGJGD alone and in combination on osteocalcin secretion of MG-63. After treated with control, anastrozole 100 μmol/L, SGJGD 12 mg/ml or anastrozole 100 μmol/L + SGJGD 12 mg/ml (A+S) for 48 h, osteocalcin secretion of MG-63 was investigated by radioimmunoassay. $^{*}P$<0.05, compared with control group.

The mRNA expression levels of osteocalcin in MG-63 cells were shown in Figure 7. Both SGJGD alone and combined with anastrozole significantly increased osteocalcin gene expression in MG-63 cells (P<0.05 vs. control group).

Effects of Anastrozole and SGJGD Alone or in Combination on OPG/RANKL mRNA Expression in MG-63 Cells

Treatment with anastrozole combined with SGJGD caused a significant increase in OPG expression in MG-63 cells (P<0.01 vs. control) (Figure 8). Anastrozole and SGJGD alone did not affect the mRNA expression of OPG. The expression of key genes associated with osteoclastogenesis was also investigated. RANKL mRNA expression was decreased by anastrozole and SGJGD alone or in combination, and only SGJGD alone and anastrozole combined with SGJGD group showed significant difference when compared with control group (P<0.05) (Figure 9).

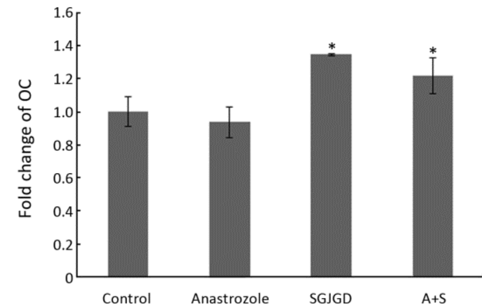

Figure 7. The levels of mRNA Expression of osteocalcin in MG-63 exposed to control, anastrozole 100 μmol/L, SGJGD 12 mg/ml or a combination of anastrozole 100 μmol/L+SGJGD 12 mg/ml (A+S). Effects of 48 h treatment were measured by real time PCR. The fold change of osteocalcin expression is calculated by the $2^{-\Delta\Delta CT}$ method. $^{*}P$<0.05, compared with control group.

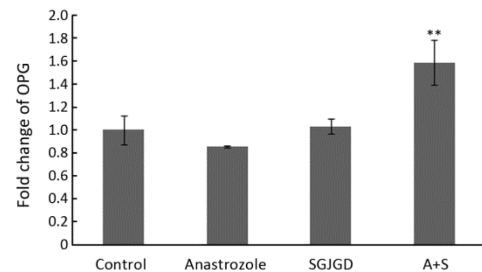

Figure 8. Effects of anastrozole, SGJGD alone or in combination on OPG mRNA expression. Real-time PCR was performed after 48 h treatment of control, anastrozole 100 μmol/L, SGJGD 12 mg/ml or a combination of anastrozole 100 μmol/L + SGJCD 12 mg/ml (A+S). The fold change of OPG expression is calculated by the $2^{-\Delta\Delta CT}$ method. $^{**}P$<0.01, compared with control group.

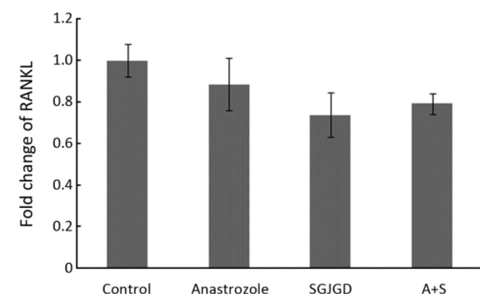

Figure 9. Effects of anastrozole, SGJGD alone or in combination on RANKL mRNA expression. After 48 h treatment of control, anastrozole 100 μmol/L, SGJGD 12 mg/ml or a combination of anastrozole 100 μmol/L + SGJCD 12 mg/ml (A+S). The expressions were measured by real-time PCR. The fold change of RANKL expression is calculated by the $2^{-\Delta\Delta CT}$ method. $^{*}P$<0.05, compared with control group.

Table 1. Primers designed for real-time PCR

Target	Primers (5′–3′)	Annealing temp (°C)	Amplification length (bp)
ALP	AGCCCTTCACTGCCATCCTGT	60	68
	ATTCTCTCGTTCACCGCCCAC		
OC	CAAAGGTGCAGCCTTTGTGTC	60	150
	TCACAGTCCGGATTGAGCTCA		
OPG	GGCAACACAGCTCACAAGAA	62	116
	CGCTGTTTTCACAGAGGTCA		
RANKL	AGG AGCAAGCTTG AAGCTCAG	62	221
	TCTCCTGAAGTTTCATGATGTCG		
GAPDH	GACCCCTTCATTGACCTCAAC	62	219
	CTTCTCCATGGTGGTGAAGA		

OC: osteocalcin

DISCUSSION

Anastrozole, an AI, reduces the effects of estrogen on breast cancer by preventing estrogen biosynthesis[13]. The suppression of estrogen production is significantly related to increase in bone turnover and induces an accelerated bone loss. Such decrease in bone mineral density caused by AIs is known as aromatase inhibitor-associated bone loss (AIBL)[14]. No treatment is specifically approved for patients with AIBL, but bisphosphonates are currently the interventions for patients, along with adequate calcium and vitamin D supplementation and a healthy lifestyle[15,16].

According to traditional Chinese medicine (TCM) theories, kidney is in charge of bone. Bone loss is related to "kidney deficiency" and treatments with "kidney reinforcing" herbs are useful[17]. Moreover, due to "homogeny of liver and kidney", bone loss is partly associated with "liver governing free flow of qi and blood". Therefore, besides "liver soothing" and "blood cooling" herbs, which are the theoretical basis of Shuganliangxue decoction for breast cancer therapy; "kidney reinforcing" herbs, such as *Rhizoma drynariae*, were added into SGJGD[18,19] to keep the bone health. *Rhizoma drynariae* can stimulate osteoblast proliferation and differentiation, increase blood calcium and phosphorus levels and enhance bone mineral density[20]. The adaption of SGJGD was aimed to reduce the adverse effects of AIs treatment on bone.

According to MTT assay, SGJGD (0.005–50 mg/ml) significantly inhibited the proliferation of ER+ breast cancer cell line MCF-7 concentration-dependently, compared with control (P<0.05) (data not shown). The results showed SGJGD inhibited growth of breast cancer cells and the IC50 was about 11.12 mg/ml. In another study, we examined estrogenic activity of SGJGD by dual-luciferase reporter (DLR) assay and bioluminescent measurements. We found that SGJGD had no estrogenic activity[21]. These results implied that SGJGD is safe for patients with breast cancer and can be integrated with other breast cancer therapy.

Osteoblast cells play a pivotal role in bone metabolism. Osteoporosis patients have an increased risk of fractures because of the low bone mineral density and altered bone micro-architecture due to decreased proliferation and mineralization of osteoblast cells[22]. As shown in this study, anastrozole inhibited cell proliferation of MG-63. But when it was combined with SGJGD, the cell proliferation was improved.

ALP is a classical biomarker of osteoblast cell differentiation. High ALP activity is required in the early stage of extracellular matrix mineralization[23]. In this research, we investigated the differentiation capacity of osteoblasts by measuring the ALP activity and mRNA expression in MG-63 cells[24]. Osteocalcin, another classical biomarker of osteoblast cell differentiation and mineralization, was also investigated[25]. The results showed that no obvious change was found in anastrozole treated cells, but the addition of SGJGD significantly increased ALP activity, stimulated osteocalcin secretion and down-regulated expressions of the both markers, showing beneficial effects on cell differentiation.

The receptor activator of nuclear factor kappa β (RANK)/RANKL/OPG system is an important signaling pathway in regulating bone metabolism and osteoclastogenesis. RANK promotes osteoclastogenesis when it binds to RANKL, which is partly produced by the osteoblasts. This process can be blocked by the binding of OPG to RANKL[26,27]. Inhibition effect of estrogen on oesteoclasts is associated with OPG and RANKL[28-30]. Up-regulation of RANKL is one of determinants of increased bone resorption induced by estrogen deficiency[29]. An imbalance in the expressions of RANKL and OPG is an underlying mechanism in cancer treatment-induced bone loss[31]. Combined anastrozole with SGJGD increased the expression of OPG and decreased the expression of RANKL, which could reduce the RANK and RANKL binding. Then osteoclast activation was blocked and apoptosis was induced[32], and thus the bone loss was controlled. But the expression of OPG seemed not to be affected by treatment with SGJGD alone; therefore, the enhanced effect of anastrozole combined with SGJGD on OPG expression deserves further research.

In conclusion, anastrozole combined with SGJGD enhanced bone formation by stimulating osteoblast cell proliferation and differentiation. Moreover, anastrozole combined with SGJGD could up-regulate OPG and down-regulate RANKL, which could inhibit osteo-clastogenesis. However, the effect of anastrozole combined with SGJGD on osteoclast should be investigated in further research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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