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Effects of Silkworm (*Bombyx mori*) Pupa Extract on the Function of Osteoblastic MC3T3-E1 Cells

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Abstract Osteoporosis is recognized as one of the major hormonal deficiency diseases, especially in menopausal women and the elderly. When the estrogen level is reduced in the body, local factors, which are known to be related with bone resorption, are increased and promote osteoclastogenesis. In our previous study, we validated the estrogenicity of silkworm pupa. In this study, we investigated the effect of silkworm pupa extract (SPE) on the function of osteoblastic MC3T3-E1 cells. SPE (10 and 50 μg/mL) significantly elevated cell viability, alkaline phosphatase (ALP) activity, and collagen content in the cells. The effect of SPE (50 μg/mL) in increasing cell viability, ALP activity, and collagen content was completely inhibited by the presence of 10^{-6} M of cycloheximide and 10^{-6} M of tamoxifen, suggesting that SPE's effect results from a newly synthesized, protein component and that it might be partly involved in estrogen action. Furthermore, we examined the effect of SPE on the H_2O_2-induced apoptosis and production of local factors in osteoblasts. Treatment with SPE (50 μg/mL) decreased the 0.2 mM H_2O_2-induced apoptosis and the production of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and nitric oxide (NO) in osteoblasts. Our data indicate that the enhancement of osteoblast function by silkworm pupa may prevent osteoporosis and inflammatory bone diseases.

Keywords: Silkworm pupa, osteoblastic MC3T3-E1 cells, apoptosis, estrogen, inflammation

Introduction

Since postmenopausal osteoporosis results from endogenous estrogen deficiency, the role of estrogens and estrogen-like agents in regulating the activities of bone cells is crucial to the prevention of this bone disease. Estrogen replacement therapy, recommended only for women who are at high risk of osteoporosis, appears to be the most effective method to reduce the rate of postmenopausal bone loss, but may be accompanied by side-effects (1). Thus, it would be most helpful to discover natural dietary substances that minimize bone loss in postmenopausal women. Phytoestrogens, such as coumestrol, genistein and daidzein, have been reported to increase alkaline phosphatase (ALP) activity and enhance bone mineralization (2). In osteoporosis, bone loss involves both increased osteoclastic bone resorption and decreased osteoblastic bone formation (3). Reactive oxygen species (ROS) enhance osteoclastic activity (4), but inhibit the differentiation of osteoblasts (5). Osteoclast-released ROS may be involved in degrading bone matrix and inhibiting differentiation of osteoblasts, resulting in the promotion of bone resorption and the inhibition of bone formation in close proximity (6).

Apoptosis occurs in a wide variety of cell types and is a major impact on the development of numerous systems (7,8). When isolated chick osteocytes were cultured with hydrogen peroxide (H_2O_2), annexin V binding, which is the earliest marker of apoptosis, was increased in a dose-dependent fashion. H_2O_2 also induced the activation of caspase-3 and the increase in cytosolic Ca^{2+} (9). Ischemia and reperfusion could promote osteocyte apoptosis through the generation of ROS (10). ROS generated by inflammatory tissue in or adjacent to bone might cause the loss of bone that is a feature of disease such as rheumatoid arthritis or periodontitis. Osteoblast apoptosis has also been shown to be important in the pathogenesis of certain metabolic bone diseases such as osteoporosis. Recent studies highlight the importance of osteoblast apoptosis in bone diseases and the use of agents such as bisphosphonates and estrogen in preventing apoptosis in osteoblasts (11).

The MC3T3-E1 pre-osteoblastic cell line is a well-accepted model of osteogenesis *in vitro* (12). Growth and differentiation factors contained in the culture medium stimulate these cells to undergo a developmental sequence that includes proliferation of undifferentiated precursors of osteoblasts, which subsequently differentiate into post-mitotic osteoblasts capable of expressing the osteogenic phenotype (13). In addition, the classical bone formation markers, such as ALP and collagen, and several other products synthesized by osteoblasts, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and nitric oxide (NO), may also be regulated by estrogen-like substances. These inflammatory mediators have been demonstrated to influence osteoclast differentiation and activation (14).

In our previous study, silkworm pupa extract (SPE) showed excellent estrogen activity (18). Therefore, we selected silkworm pupa based on its stimulatory action on MCF-7 cells and its high estrogen receptor binding capacity. In the present study, we examined the effect of SPE on the differentiation of osteoblastic MC3T3-E1 cells *in vitro*, and investigated the oxidative stress-induced apoptosis and the production of TNF-α, IL-6, and NO in osteoblasts.

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Materials and Methods

Extraction SPE, prepared from the pupa of silkworms (*Bombyx mori*; Kunwha Pharmaceuticals, Korea), was obtained by 3-stage extraction with 70% ethanol (in water, v/v) at room temperature. The extract was filtered and concentrated in a rotary evaporator at temperature below 50, and then freeze dried (yield: 3.4%, w/w).

Cell culture MC3T3-E1 cells (RCB1126, an osteoblast-like cell line from C57BL/6 mouse calvaria), obtained from the RIKEN Cell Bank (Tsukuba, Japan), were cultured at 37°C in 5% CO_2 atmosphere in α-modified, minimal essential medium (α-MEM; GIBCO, Gaithersburg, MD, USA). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin.

Cell viability The cells were suspended in medium and plated at a density of 7.0×10^3 cells/well into a 96-well culture dish (Costar, Cambridge, MA). After 24 hr, the medium was replaced with phenol red-free media containing 5% charcoal-dextran-treated FBS (CD-FBS) supplemented with glabridin. After 2 days of culture, cell proliferation was measured by MTT assay based on the ability of viable cells to convert soluble 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble, dark blue, formazan reaction product. In the bulk cell photometric MTT assay, the bulk conversion of MTT in the well plate was measured photometrically. MTT was dissolved in phosphate buffered saline (PBS) at a concentration of 5 g/L and sterilized by passage through a 0.22 mm filter. This stock solution was added at one part to 10 parts medium to each well of the culture plate, which was then incubated at 37°C for 2 hr. Dimethylsulfoxide was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader at a wavelength of 570 nm.

Alkaline phosphatase (ALP) activity After the cells were cultured at a density of 10^6 cells/culture dish for 7 days, the medium was replaced with phenol red-free α-MEM containing 5% CD-FBS. Then, the cells were cultured with the sample in the presence of 10 mM β-glycerophosphate (β-GP), which was added to initiate *in vitro* mineralization (19). After 3 days, the medium was removed and the cell monolayer was gently washed twice with PBS. The cells were lysed with 0.2% Triton X-100 and the lysate was centrifuged at 14,000×*g* for 5 min. The clear supernatant was used for the measurement of ALP activity and protein concentration. ALP activity and protein concentration were determined using an ALP activity assay kit (Wako, Tokyo, Japan) and a BCA-protein assay kit (Pierce, Rockford, IL), respectively.

Collagen contents After the cells were cultured at a density of 10^6 cells/culture dish for 7 days, the medium was replaced with α-MEM containing 5% CD-FBS. Then, the cells were cultured with sample in the presence of 10 mM β-GP for 3 days and cellular collagen content was measured using a Sircol Collagen Assay kit (Biocolor Ltd., Newtownabbey, Northern Ireland). This kit is a quantitative dye-binding method designed to analyze collagens extracted from mammalian tissues and cells during *in vitro* culture. The dye reagent binds specifically to the $[Gly\text{-}X\text{-}Y]_n$ helical structure found in mammalian collagens (types I to V).

Quantitation of apoptosis in MC3T3-E1 cells A TiterTACS kit (R&D System Inc., Minneapolis, MN, USA) was employed to assess apoptosis in osteoblastic cells. The kit provides quantification of apoptosis in cultured cells without direct counting of labeled cells and without detecting DNA fragmentation in cells grown as a monolayer. After incubation with sample and 0.2 mM H_2O_2, the cells were harvested, fixed and labeled according to the TiterTACS protocol prior to colorimetric analysis. Cells were incubated with TACS-Sapphire substrate and the colorimetric reaction was stopped with 2 N HCl after 30 min.

Cytokines (TNF-α and IL-6) immunoassay After the cells were treated with sample and 0.2 mM H_2O_2 for 48 hr, TNF-α and IL-6 contents in the medium were measured with an enzyme immunoassay system (R&D System Inc., Minneapolis, MN, USA) according to the manufacturer's recommendation. In brief, cytokine present is bound by immobilized antibody pre-coated onto a microplate. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for cytokine is added to the well. After washing to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when stop solution is added. The intensity of the color measured is in proportion to the amount of cytokine bound.

Determination of nitrite production After cells were treated with sample and 0.2 mM H_2O_2 for 48 hr, nitrite production, an indicator of NO synthesis, was measured in the culture supernatant of osteoblasts, as described previously by Kleinerman *et al.* (20). Briefly, after mixing 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) with 100 μL culture supernatant, optical density at 540 nm was measured by using a microplate reader. Nitrite concentrations were calculated from the standard curve of sodium nitrite prepared in culture medium.

Statistics The results are expressed as mean ± SEM (n=6). Statistical analysis was performed using one-way ANOVA ($p<0.05$). The analysis was performed using SAS (V8, SAS Institute, North Carolina, USA) statistical software.

Results and Discussion

The use of silkworm pupa in the treatment for hormone-related symptoms such as impotence in Korea prompted us to investigate whether silkworm pupa might exhibit estrogenic activity, a property that is known to produce a protective benefit on osteoporosis. In our previous study,

SPE had a significant binding capacity to the estrogen receptors and proliferation-inducing activity in MCF-7 cells (18). To investigate the effect of SPE on bone metabolism, we employed a cell culture system in this study. MC3T3-E1 cells, derived from newborn mouse calvariae, display osteoblast-like characteristics (21). ALP and collagen are the most widely recognized biochemical markers for osteoblastic activity. Therefore, we examined the effect of SPE on the cell growth, ALP activity, and collagen synthesis of osteoblastic MC3T3-E1 cells.

The effect of SPE on the growth of osteoblastic MC3T3-E1 cells is shown in Fig. 1. Osteoblast growth was elevated significantly by the presence of SPE (4~250 μg/mL) (Fig. 1A). When cells were cultured in a medium containing either vehicle, SPE (50 μg/mL), cycloheximide (10^{-6} M) plus SPE (50 μg/mL), or tamoxifen (10^{-6} M) plus SPE (50 μg/mL), the SPE-induced increase in cell survival was completely inhibited by the presence of cycloheximide or tamoxifen (Fig. 1B). ALP activity was measured to study the effect of SPE on the osteoblastic differentiation in MC3T3-E1 cells (Fig. 2). Culture in the presence of SPE (10 and 50 μg/mL) caused a significant increase in the ALP activity of osteoblastic cells (Fig. 2A). However, the presence of cycloheximide (10^{-6} M) or tamoxifen (10^{-6} M) eliminated the effect of SPE (50 μg/mL) in increasing ALP activity (Fig. 2B).

The effect of SPE on collagen content in osteoblastic MC3T3-E1 cells is shown in Fig. 3. Cells were cultured in a medium containing SPE. The presence of SPE (10 and 50 μg/mL) significantly increased collagen content (Fig. 3A). The SPE (50 μg/mL)-induced increase in collagen synthesis was clearly eliminated by the presence of cycloheximide (10^{-6} M) or tamoxifen (10^{-6} M) (Fig. 3B). On the basis of these data, SPE appears to stimulate the proliferation and differentiation of osteoblasts. These results also support the stimulatory nature of SPE toward the function of osteoblastic cells. On the other hand, the stimulatory effects of SPE on cell growth, ALP activity, and collagen synthesis were clearly blocked by the presence of cycloheximide (protein synthesis inhibitor) and tamoxifen (anti-estrogen reagent). These results suggest that the effect of SPE (50 μg/mL) in increasing bone formation results from a newly synthesized protein component such as collagen and ALP, and that it is mediated through an estrogen-like action. This observation is in agreement with the findings of a previous study suggesting that the anabolic effect of soybean phytoestrogen in osteoblastic MC3T3-E1 cells was completely inhibited by the presence of cycloheximide and tamoxifen (22).

Jeong *et al.* (23) reported that Drynariae Rhizoma promotes osteoblast differentiation and mineralization in MC3T3-E1 cells through regulation of bone morphogenetic protein-2, ALP, type I collagen and collagenase-1. We

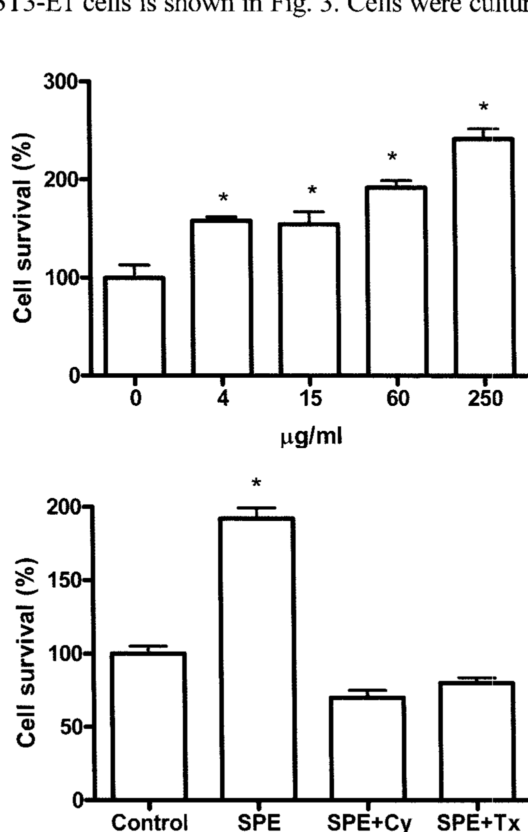

Fig. 1. Effect of SPE on the viability of MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence or absence of SPE (Fig. 1A), and in combination with 50 μg/mL SPE and 10^{-6} M cycloheximide (Cy) or 10^{-6} M tamoxifen (Tx) (Fig. 1B) for 48 hr. Data shown are mean ± SEM, expressed as a percentage of control. The control value for MTT assay was 0.247 ± 0.004 OD. *p<0.05 vs. control.

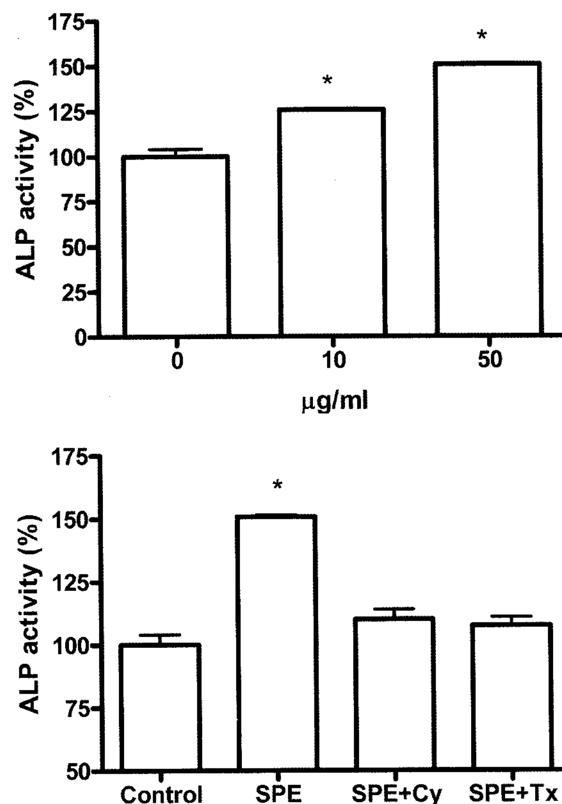

Fig. 2. Effect of SPE on the alkaline phosphatase activity of MC3T3-E1 cells. After the cells reached confluence, the medium was replaced with phenol red-free α-MEM containing 5% CD-FBS in the presence or absence of SPE (Fig. 2A), and in combination with 50 μg/mL SPE and 10^{-6} M cycloheximide (Cy) or 10^{-6} M tamoxifen (Tx) (Fig. 2B). Data shown are mean ± SEM, expressed as a percentage of control. The control value for ALP activity was 1.88 ± 0.09 Unit/mg protein. *p<0.05 vs. control

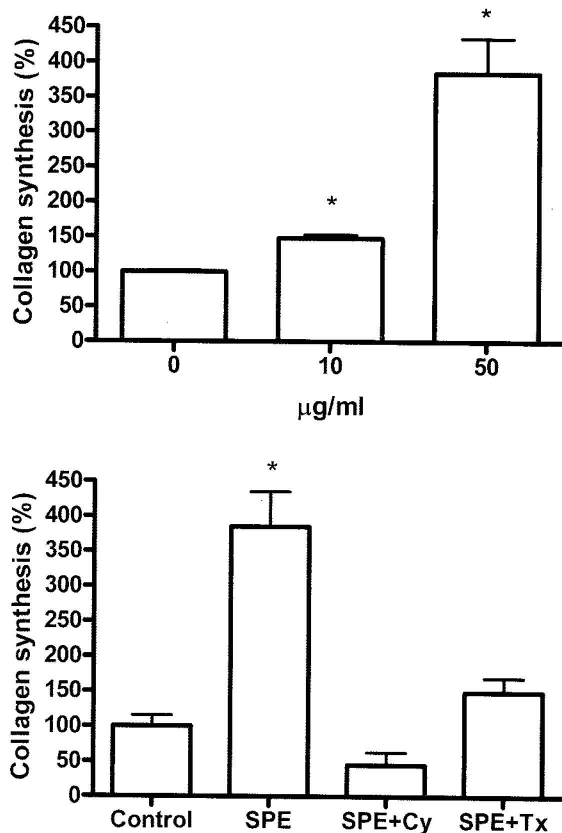

Fig. 3. Effect of SPE on the collagen synthesis of MC3T3-E1 cells. After the cells reached confluence, the medium was replaced with phenol red-free α-MEM containing 5% CD-FBS in the presence or absence of SPE (Fig. 3A), and in combination with 50 μg/mL SPE and 10^{-6} M cycloheximide (Cy) or 10^{-6} M tamoxifen (Tx) (Fig. 3B). Data shown are mean ± SEM, expressed as a percentage of control. The control value for collagen content was 2.83 ± 0.43 μg per 10^7 cells. *$p<0.05$ vs. control.

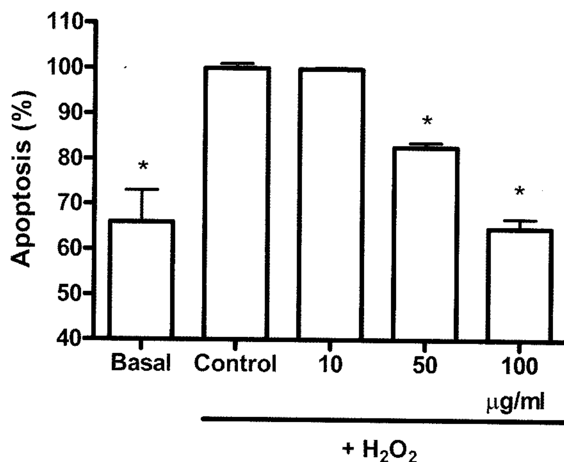

Fig. 4. Effect of SPE on MC3T3-E1 cell apoptosis induced by H_2O_2. Cells were cultured with SPE in the presence of 0.2 mM H_2O_2. Basal: cells not treated with H_2O_2. Control: cells treated with H_2O_2. Apoptosis was assessed by cell death ELISA. Data shown are mean ± SEM, expressed as a percentage of control. The control value for apoptosis assay was 0.27 ± 0.01 OD. *$p<$ 0.05 vs. control.

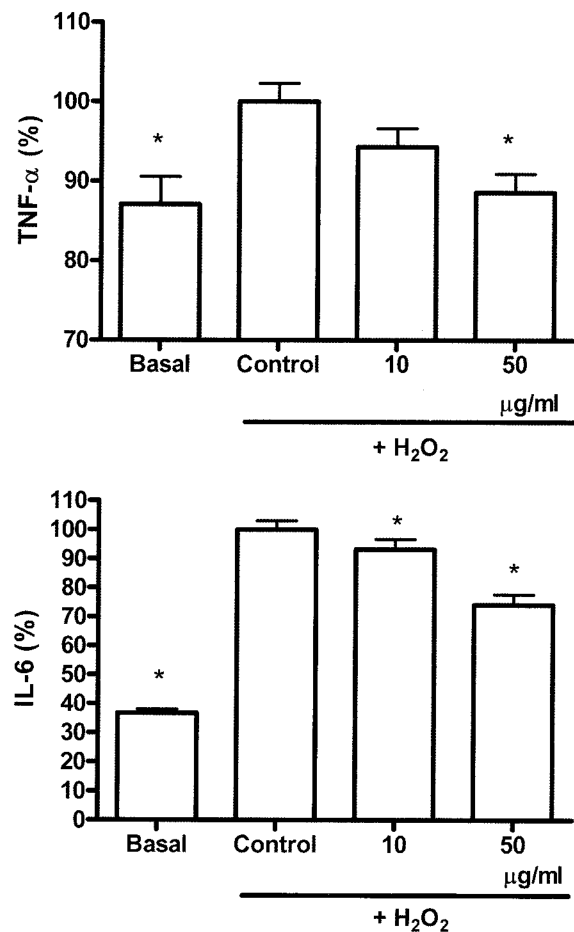

Fig. 5. Effect of SPE on H_2O_2-induced cytokines production of MC3T3-E1 cells. MC3T3-E1 cells were cultured with vehicle or SPE in the presence of 0.2 mM H_2O_2 for 48 hr. Basal: cells not treated with H_2O_2. Control: cells treated with H_2O_2. TNF-α and IL-6 concentrations were measured in the conditioned medium. Data shown are mean±SEM, expressed as a percentage of control. The control values for TNF-α and IL-6 production were 7.45 ± 0.17 pg/mL and 12.77±0.69 pg/mL, respectively, per 10^5 cells. *p <0.05 vs. control.

investigated whether SPE modulates osteoblast apoptosis and production of local factors induced by H_2O_2. To determine the effect of oxidative stress on osteoblasts, we treated the cells with H_2O_2 and quantitated the apoptosis induced by H_2O_2 in osteoblastic MC3T3-E1 cells (Fig. 4). When MC3T3-E1 cells were treated with 0.2 mM H_2O_2, apoptosis was increased. Treatment with SPE (50 and 100 μg/mL) significantly inhibited H_2O_2-induced apoptosis in MC3T3-E1 cells.

We investigated whether SPE modulates osteoblast production of TNF-α and IL-6 (Fig. 5), and of NO (Fig. 6). When 0.2 mM H_2O_2 was added to cells, production of TNF-α, IL-6 and NO all increased significantly. However, treatment with SPE at 50 μg/mL significantly inhibited H_2O_2-induced TNF-α and IL-6 production, while SPE treatment at 50 and 100 μg/mL significantly inhibited H_2O_2-induced NO production. Our data indicate that SPE inhibits apoptosis in osteoblasts, which demonstrates that the stimulating effect of SPE on osteoblastic MC3T3-E1 cells may be mediated, at least in part, by the inhibition of apoptosis. However, additional studies are clearly needed to further define the mechanism of SPE on apoptosis in osteoblasts, as well as the anti-osteoporotic effect of SPE

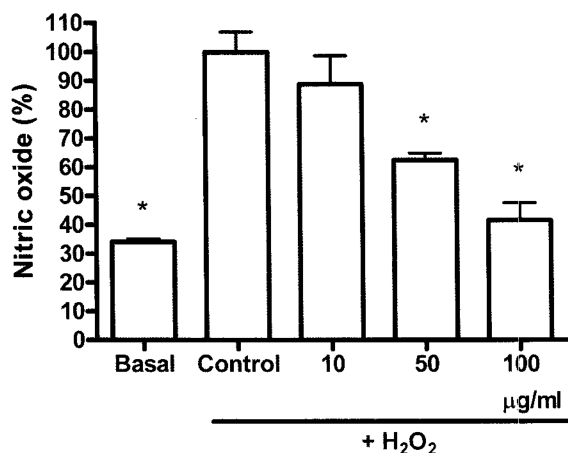

Fig. 6. Effect of SPE on H_2O_2-induced NO production of MC 3T3-E1 cells. MC3T3-E1 cells were cultured with vehicle or SPE in the presence of 0.2 mM H_2O_2 for 48 hr. Basal: cells not treated with H_2O_2. Control: cells treated with H_2O_2. Nitrite concentration was measured in the conditioned medium. Data shown are mean ± SEM, expressed as a percentage of control. The control value of NO production was 47.06 ± 3.27 mM per 10^5 cells. *p<0.05 vs. control.

and related compounds.

Hata *et al.* (24) isolated (22*E*,24R)-ergosta-7,22-diene-3β,5α,6β-triol from the mushroom as an active compound from the fruiting bodies of *Tricholoma auratum* and found that it has a protective effect against the MC3T3-E1 cell apoptosis induced by serum starvation. Moreover, Park *et al.* (25) reported that the H_2O_2-induced reductions of proliferation and differentiation were inhibited by pre-incubating the osteoblasts with green tea polyphenols. ROS might also indirectly stimulate osteoclasts by augmenting the expression of resorptive cytokines such as TNF-α and IL-6 that have been strongly implicated in estrogen-deficiency bone loss (26). ROS are potent inducers of these cytokines in many cells through activation of NF-κB (27). Therefore, ROS might induce bone hyper-resorption in estrogen deficiency through the autocrine-paracrine effects of ROS-augmented TNF-α expression.

Previous studies have shown that TNF production by peripheral blood monocytes was increased in women after natural or surgical menopause, and that this increase was blocked by estrogen replacement (28). NO plays an important role as a mediator of diverse cellular functions in a variety of cell types (29) and is also thought to play a role in osteoblast function. Inflammatory mediators may induce inducible NO synthase expression in osteoblasts, and NO may be responsible for the suppression of bone formation.

These results presented above indicate that SPE might be useful for diseases associated with the excessive production of ROS, and that skeletal tissues may benefit from the consumption of silkworm pupa. Estrogens have been shown to promote survival and differentiation of several cells maintained in culture, to reduce cell death associated with excitotoxicity, oxidative stress, or serum deprivation, and to reduce levels of inflammatory mediators, cytokines and chemokines. Although the cellular and molecular mechanisms implicated in the protective actions of estrogen on the bone are not completely understood, it is suggested that modulation of cell death regulators, such as Bcl-2, Akt and calpain, as well as interaction with growth factors and their receptors, is relate to these mechanisms (30). In addition, Yu *et al.* (31) also demonstrated that estrogen quenches H_2O_2-induced up-regulation of apoptosis-related protein, and protects cell degeneration, probably through caspases, extracellular matrix proteins, metabolism pathway components, GTP/GDP exchangers, G-protein GTPase activity modulators, transcription activators and repressors.

In summary, the data described in this report combine to suggest that silkworm pupa exerts an effect on osteoblast-like cells and that its beneficial effects are mediated through estrogen receptors and protein synthesis. In addition, the inhibition of H_2O_2-induced apoptosis and TNF-α, IL-6, and NO production by silkworm pupa may be partly related to the inhibition of osteoclastogenesis.

Acknowledgments

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