In Vitro Study of the Protective Effects of Hydroalcholic Extract of Soybean against Impact of Oxidative Damage on Osteogenesis and Chondrogenesis of Mouse Limb Bud

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ABSTRACT

Introduction: Oxidative stress has been implicated in the pathogenesis of various diseases affecting chondrogenesis or the function of articular cartilage. The purpose of the present study was to find the effect of soybean extract on reduction of detoriation effects of oxidativestress in embryonic chondrogenesis in vitro.

Methods: In order to separate ectoderm from mesenchyme, the limb buds of mouse embryos (12-13days) removed and incubated in dispase. After separation, the limb bud rinsed and incubated in the trypsin. Adding culture media (DMEM/F12 contain 10% FBS) and pipetting, the limb buds were dissociated. The number of the cells adjusted to 1-2 $\times 10^7$/mL. Superconfluent micro mass spotting of cell suspension in 96 well tissue culture plate were formed. After incubation for 1.5-2 h at 37 ºC and 5% CO_2, the plate flooded with culture media. At day 5, different concentration of H_2O_2 and soybean extract added to fresh media and in order to induce oxidative stress, incubated for 24h. The cultures were stained with alcian blue to prove the cartilage Differentiation and alizarin red for calcium deposit.

Results: The results indicated that cell viability diminished by extract and H_2O_2 administration; although, the supplementation of the cells exposed to the oxidative stress with the extract improved cell proliferation rate. The soybean also improved the ossification and chondrification of the cells exposed to H_2O_2.

Conclusion: The study demonstrated that soybean extract can reduce the effect of oxidative stress in embryonic chondrogenesis.

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1. Introduction

About 50 years ago, the oxidative stress suggested being the cause of aging process and age-related degenerative disorders (1). Gene expression, regenerative capacity and apoptosis can be influenced by free radicals such as H_2O_2 produced during normal metabolic process in the cells (2). Oxidative stress begins when the amount of free radicals are more than the ability of the cells to scavenger them (3). It also plays roles in the process of bone loss including osteoporosis. Free radicals formed during the normal function of osteoclast can stimulate the bone matrix resorption (4). Overwhelming of free radicals more than the capacity of the cells to neutralize them lead to extensive bone loss and subsequently, bone fracture and osteoporosis (5). Antioxidant content in the diet demonstrated to prevent the bone loss due to oxidative stress and improve the skeletal disorders (6).

Soybean is a rich source of isoflavones such as genestein (7). Soybean isoflavones appear in the plasma and urine (8). A meta-analysis of randomized controlled trials showed that intake of isoflavones containing soybean extract by postmenopausal women led to an increase in the bone mineral density of the lumbar spines (9). Long-term human trials also revealed positive effects of soybean on bone mass in premenopausal women (10). However, the positive effects of soy bean isoflavones on bone loss as a menopausal symptom is controversial (10, 11). Also, in the skeletal development of mouse embryo, the positive effect of the soybean extract was shown (12). Soy isoflavones with estrogenic potential and its chemical structure showed a similarity to estrogen (13). Therefore, the effect of the soybean on prevention of bone loss can be attributed to the estrogenic activity.

Soybean extract showed antioxidant properties (14). The effects of maternal feeding of the soybean isoflavones on oxidative stress was also demonstrated in a 12-month randomized double blind controlled trial (15). Prenatal exposure of the embryo with soybean isoflavones such as genestein was shown to protect the liver DNA from oxidative stress damages (16). The soybean extract also reduced the cell proliferation as well as apoptosis in a dose dependent manner (17). It was found that in the rats deprived from soybean in their diets, oxidative stress increased (18).

Due to both antioxidant and estrogenic properties, soybean influences osteogenesis and chondrogenesis. The isoflavones of the soybean such as genestein can be concentrated in placenta (19) and cross the blood-placental barrier and appeared in the fetal tissues (20). The purpose of the present study was to evaluate the effects of soy extract on prevention of the damages caused by oxidative stress.

2. Materials & Methods

2.1. Extract Preparation

Soybean seeds obtained from Seed Improvement Institute, Iran. Using percolation method, the water/alcoholic extract was prepared. The soybean were dried and powdered. 50 g of the dried powder was suspended in water / alcohol and percolated for 72 h. The extract yield was 25 g powder used for cell culture.

2.2. Cell Culture

The guide lines of the ethic for handling of animals approved by Shiraz University of Medical Sciences and applied for all animal treatments. The pregnant mice, at 12 day of gestation, sacrificed by cervical dislocation and then the embryos removed. The upper and lower limb buds sectioned and collected in phosphate buffer saline (PBS). The limb buds exposed to 1U dispase (Gibco, UK) for 1.5 h at 37°. Dispase digested the basement membrane and separated ectoderm from mesoderm. The mesoderm treated with 1% trypsin (Sigma) for 20 min at 37°. Using culture medium containing fetal bovine serum (FBS), the trypsin was neutralized. Afterwards, using mild pipetting, the cells dissociated. Using trypan blue and hemocytometer, the number of viable cells was counted and adjusted to 2.5×10^7 cell/ mL. Then, 20µL of the cell suspension plated on each well of a 24 well culture plate and incubated for 1.5 h at 37 °. Afterwards, DMEM/F12 containing 10% FBS, L-glutamine and penicillin/ streptomycin added to the cells and incubated at 37° and 5% CO_2.

2.3. Experimental Design

The cultures divided into10 groups. The control cells cultured without intervention. The second groups of the cultures received 10mM H_2O_2. The third groups of the cultures exposed to the 10, 100, 1000 and 10000 µg/ mL of the extract. The forth group received 10mM H_2O_2 with 10, 100, 1000 and 10000 µg/ mL of the extract. Using osmometer, the osmolarity of the extract contained culture media were measured. After 24 h of the beginning of the experiment, the cells were treated with soybean extracts. All experiments performed in triplicate.

2.4. Viability Test

The culture media replaced with 500 µL of 0.005% neutral red in normal saline and incubated at 37 centigrade for 2 h. Then, the cells fixed with calcium formol for 1 min. Adding acid /alcohol (5% acetic acid in 50% ethanol) for 2 h, the dye was eluted. The optical density of the eluted dye evaluated at the wavelength of 550nm.

2.5. Cartilage Differentiation Assay

The cultures washed with PBS and fixed with Kahl's fixative. After washing, the cells stained with 5% alcian blue, pH 2. The cultures washed with ethanol and then with PBS. The cells photographed with stereomicroscope. The nodules stained with alcian blue represented the foci of chondrification.

2.6. Bone Differentiation Assay

The cultures washed with PBS and fixed by methanol. The cells stained with 5% alizarin red S at room temperature. Then, the cells washed in water and photographed with a stereomicroscope.

3. Results

After 1.5 h of micromass formation, the cells attached to the plate. In the control cultures, the cells form nodules which were round and impact aggregates of the cells. Each nodule contained the cells that involved in matrix secretion. Adding H_2O_2, the cells exposed to the oxidative stress failed to attach to the plated surface and form nodules but the cells exposed to the soybean attached to the plate surface and formed nodules (Fig 1).

Cell viability test showed that supplementation of the cells with 10000µg/mL of soybean extract, with or without H_2O_2, led to a significant reduction in cell viability (for both P= 0.000). Administration of the 1000µg/mL of soybean extract also reduced the cell viability significantly (P= 0.001). The soybean supplementation improved cell viability in a dose-dependent manner so that the lower concentration of the soybean extract led to the highest viability among the cultures treated with soybean. However, none of the soybean-treated cultures could contain the viable cells as high as the control cultures. The lowest number of the viable cells belonged to the group treated with both 10000µg/mL of soybean

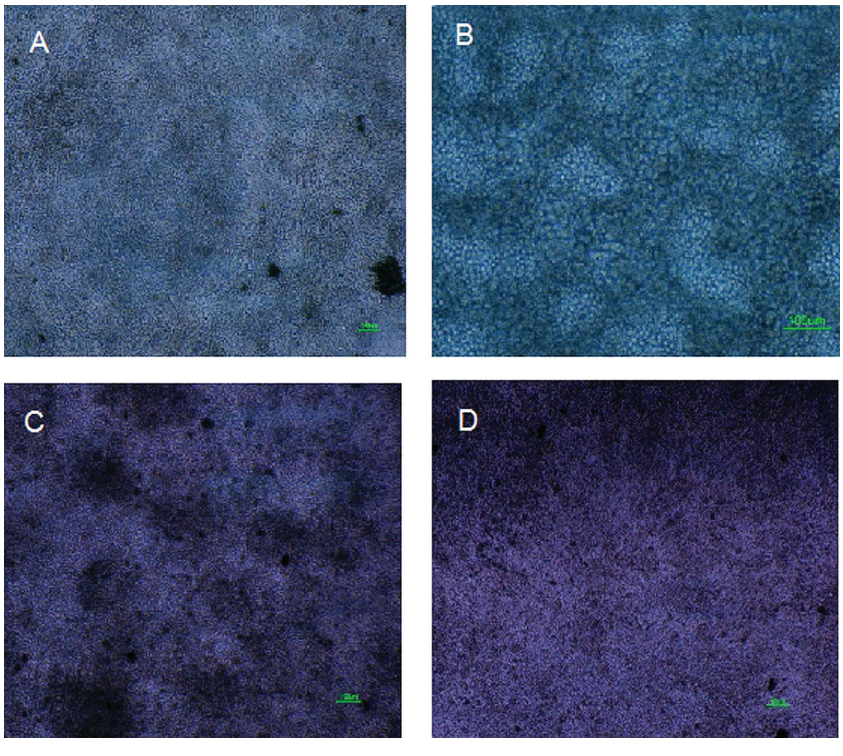

Figure 1. The inverted microscopy of the limb bud mesenchymal cells. The nodules can be observed. A, control; B, soybean treated (10µg/mL); C, soybean and H_2O_2 traded (1000µg/ mL); D, H_2O_2-treated cultures.

extract and H_2O_2. Except for 10000µg/mL of the extract, the soybean improved the H_2O_2 toxicity in a dose-dependent manner. The administration of the 1000 and100 µg/mL of the extract showed a significant increase in cell growth (P= 0.005 and P= 0.014, respectively) (Fig 2).

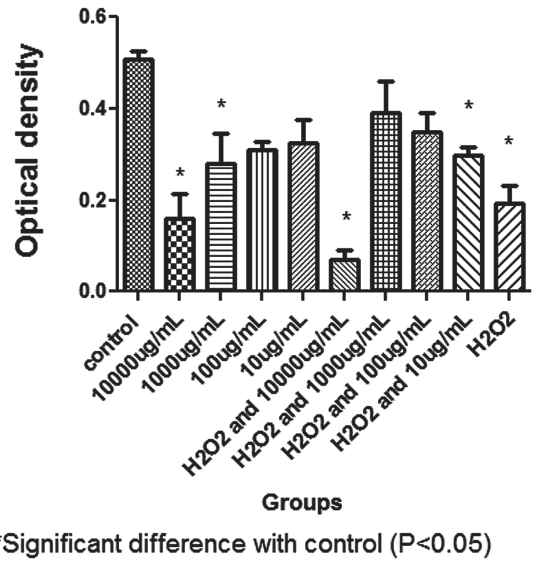

Figure 2. the comparison of the cell viability after treated the cells with oxidative stress and soybean extract.

Alcian blue staining showed that the number of the nodules expressed proteoglycan reduced after extract administration. However, both alcian blue and alizarin red S staining assays revealed that soybean extract formed nodules with larger diameters. After soybean supplementation, the amount of Ca^{+2} resorption was also increased. H_2O_2-exposed cells could not form any nodules. However, the treatment of the cultures exposed to oxidative stress with H_2O_2 improved the capability of the cells to form nodules and secreted proteoglycan (as an indicator of cartilage formation) and ca^{+2} deposition (as an indicator of bone formation) (Fig 3).

4. Discussion

Soybean contains flavonoids with different effect on cell proliferation rate in a dose-dependent manner (21). It was shown that soybean flavonoid, genestein, exerts a dual effect on cell proliferation; it induced cell growth at lower level and cell apoptosis at higher level (22). In addition, the effects of various flavonoid-containing extracts on cell viability and cell proliferation have been shown in spermatogenesis (23) and osteogenesis (24) previously. The results of the present study showed that as the extract concentration decreased, the cell viability increased. The administrations of 10000µg/mL of soybean extract reduced cell viability and could not improve the effects of oxidative stress after exposing the cultures

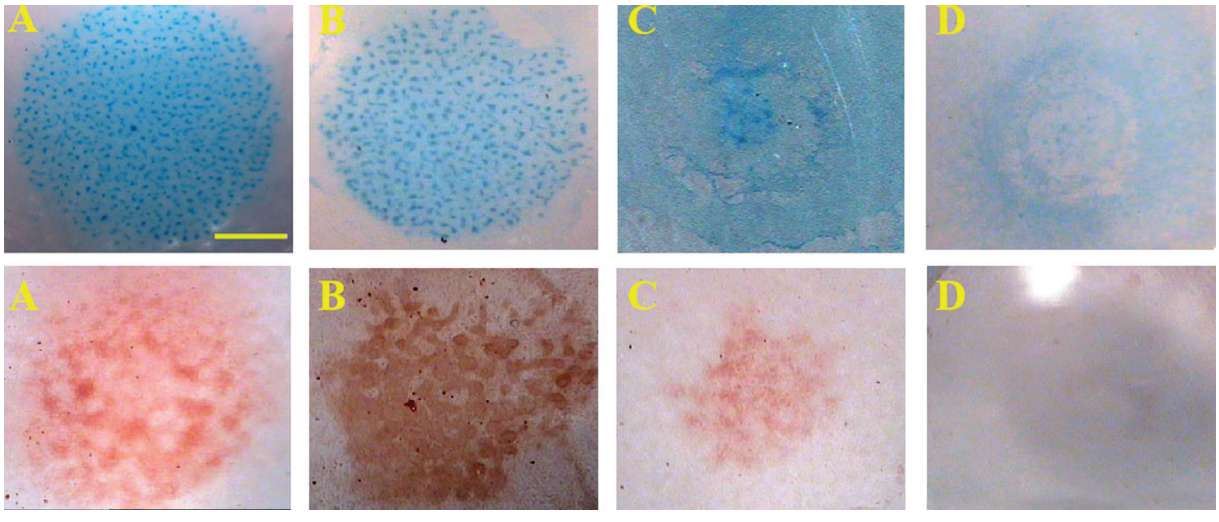

Figure 3. The above raw shows alcian blue staining and below raw shows the alizarin red S staining of the limb bud cultures. Control (A), soybean treated (10µg/mL) (B), soybean and H_2O2 traded (1000µg/ mL) (C) and H_2O_2-treated cultures (D). Scale bar is 1 mm.

to H_2O_2. The high concentration of the soybean extract was toxic. Although, the cell viability could not improve after soybean extract administration; it improved the toxic effect of H_2O_2 in a dose-dependent manner. In prevention of the toxicity exerted by H_2O_2, it was found that the higher concentration of the soybean extract was more effective.

Soybean contains isoflavones such as genestein. genestein acts as agonist or antagonist of estrogen (25). Cartilage development inhibited by estrogen-dependent pathway (26). Genestein inhibited the synthesis of extracellular matrix and proliferation of the chondrocytes (27). The results of this study also showed that the extract-treated cultures contained less number of nodules; however, the diameter of each one was larger than in non-treated cultures. Apparently, the extract led to a decrease in cell differentiation toward chondrogenic lineage. The extract-treated cultures showed larger area of ossteogenesis. It was shown that bone loss improved by genestein administration (28). Genestein also exerted osteogneic activity (29).

It was shown that the capability of chondrocytes to produce proteoglycan and collagen influenced by free radicals (30). This study also showed the detrimental effects of H_2O_2 on chondrogenesis and osteogenesis. Both proteoglycan and Ca^{2+} deposition were affected by H_2O_2 administration. On the other hand, the flavonoid content of the soybean improved osteogenesis. After soybean extract administration, the improvement of the oxidative stress status in rat uterus was observed (31). In osteoblastic cell line, the scavenger activity of soybean extract for removal of free radicals was shown (32). It was also showed that supplementing culture media of limb bud mesenchymal cells with hydroalcholic soybean extract improved both viability and capability of the cells to differentiate toward osteogenic and chondrogenic cell lineages.

In conclusion, hydro/alcoholic soybean extract could not increase cell proliferation rate but it induced the cells to form larger nodules containing calcium deposition. Nevertheless, soybean extract could act as free radical scavenger and adding it to the culture media improved the culture condition for cell growth and differentiation.

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