Original Article

The Effect of *Cerastoderma lamarcki* and Rice Bran Extract on Wharton's Jelly-Derived Mesenchymal Stem Cells Differentiation

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Abstract

Background & Objective: Nowadays, the use of natural materials in regenerative medicine is very important, especially using substances with calcium carbonate structure due to their similarity to the bone structure. In this study, the effect of *Cerastoderma lamarcki* shell and rice bran extract on stem cell proliferation and differentiation has been investigated.

Materials & Methods: First, *crastoderma* shells were collected and cut into small pieces. Its compounds were analyzed by X-ray diffraction. Finally, the structural features of shells were investigated by SEM. Stem cells were extracted and cultured from the umbilical cord of Wharton's jelly. After cells seeding on the scaffold, cell survival was studied by MTT. Adhesion, morphology, and diffusion of cells on the shells were also examined by SEM. Cells were stimulated by osteogenic medium and osteoblastic differentiation by alkaline phosphatase activity was studied. Rice bran ethanolic extract was used. Cell survival was studied by MTT technique and osteogenic differentiation was studied by Alizarin Red staining.

Results: MTT studies showed appropriate adhesion of cells on the scaffold and SEM studies also showed successful binding and their appropriate morphology on the scaffold. Alkaline phosphatase studies showed that osteogenic differentiation of cells on shells is significant. In another part of this study, we studied the survival and osteogenic differentiation of these cells in treatment with ethanolic extract of rice bran. MTT studies showed a dose-dependent decrease in cell survival in the presence of the extract. Alizarin staining also showed the differentiation potential of this substance.

Conclusion: The results showed the appropriate potential of these two natural substances in bone differentiation. However, further studies are needed to prove their effects.

Keywords: bone tissue engineering; *Cerastoderma lamarcki* shell; Wharton's jelly derived mesenchymal stem cells; rice bran extract

Introduction

A Repair of bone defects caused by severe trauma, resection of tumors, and congenital deformity has been a serious challenge to surgeons. Because of its various osteogenic, osteoinductive, and osteoconductive properties, autogenous bone graft is recognized as a standard candidate for bone grafting. However, increasing incidence of morbidity during autogenous graft harvest might not be as desirable as expected (1, 2). To overcome this problem, tissue engineering has emerged as a promising approach to treating the diseased bone. The most common approach to bone tissue engineering is based on developing new biomaterial scaffolds to provide a suitable environment for proliferation, differentiation and induction of tissue regeneration(3). Needless to say, mesenchymal stem cells (MSCs) have the potential to be differentiated into osteoblasts for

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bone tissue engineering applications (4). Generally, several biomaterials can be used as scaffolds in bone engineering, such as fibrin and demineralized bone (5). Natural materials have been of interest to many researchers for years(6, 7). They have been extensively used in stem cell biology as natural/biological scaffolds and as stem cells regulators (8). In the present research, we aimed to study the effect of two natural materials on mesenchymal stem cells. One of the most interesting aspects of our choice of natural products is that one of them is animal-based and the other one is plant-based.

Special attention is recently paid to marine origin materials which are enormously diverse. The marine environment is a rich source of organisms with unique bone-like structures (9). Among them, coral materials are considered effective biomaterials in bone tissue engineering due to their properties of osteoconduction, biodegradability, and biocompatibility (10). *Cerastoderma lamarcki* is one of the bivalves whose shell can be found in abundance in Caspian Sea coasts. Although, there are not enough data about this bivalve and its potential in bone tissue engineering, several studies have reported the use of calcium carbonate from natural aragonite as a potential bone substitute for bone regeneration (11-13). Thus, natural *Cerastoderma* shell might be used as a bone substitute without processing its calcium carbonate to hydroxyapatite. Therefore, the aim of our research is to evaluate the biocompatibility, attachment, proliferation and osteoblastic differentiation of mouse umbilical cord Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) cultured on *Cerastoderma shell*.

In another phase of our study, we investigated the effect of rice bran extract (RBE) on proliferation and osteoblasts differentiation of WJ-MSCs. Although rice bran is an agricultural by-product, it has been reported that it contains various nutrients such as vitamins, minerals and amino acids with many health benefits (14). It is also shown that among different bioactive compounds in rice bran, several substances such as Ferulic acid, Hydroxycinnamic acid, Tocopherols, and Tocotrienols can inhibit osteoclastogenesis and prevent bone loss. Thus, it has been illustrated that rice bran can reduce the differentiation potential of osteoclast cells via downregulation of MAP kinase activity and c-Fos expression (15).

Materials & Methods

1. Preparation of *Cerastoderma* shell blocks and its characterization by X-ray diffraction

Cerastoderma shells were harvested from Caspian Sea coast (Sari coast), gently washed and dried. They were soaked in NaOH2N for 5 minutes, then treated with H2O2 30% for 10 minutes to remove trapped particles (debris and organic remnants). They were then broken into small pieces and shaped with sandpaper to obtain flat squared shell scaffolds. The samples were cut into blocks of 1mm in thickness and 15 mm in diameters. The prepared *Cerastoderma* shells were rinsed with distilled water and sterilized in an autoclave at 121 ^{0}C for 40 min (9, 10). The mineralogy of shells was scrupulously verified as follows: small fragments of clean shells were powdered in an agate mortar. X-Ray Diffraction (XRD) pattern was analyzed by X-ray diffractometer (LE01430VP, England)(16).

2. Mesenchymal stem cell culture

MSCs were derived from mouse umbilical cord Wharton's Jelly. NMRI mice in the last week of gestation (E18-E21) were sacrificed by cervical dislocation and the umbilical cords of embryos were removed. After mechanical and chemical digestion, the umbilical cords were transferred into 25 ml flasks with 5 ml DMEM medium containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin G: 10,000 units/ml, amphotericinB:25 µg/ml; Gibco-BRL) at 37 ^{0}C and 5%CO2. After two days, umbilical cord pieces adhered to flask and WJ-MSCs began to expand from them (17).

3. Characterization of WJ-MSCs

WJ-MSCs were treated with 0.25% trypsin and washed with PBS. They were fixed in 4% formalin and permeabilized with Triton x-100 for 15 minutes at room temperature. Then, in the presence of anti-Oct-4 antibody cells were kept in refrigerator for 10 hours. Unbound antibodies were removed by washing with PBS. Following a 10- min centrifugation with FITC conjugated secondary antibody at 2000rpm, the cells were resuspended in 1% formalin and analyzed with a flow cytometer (16).

4. Implantation of cells on *Cerastoderma Lamarcki*

On seventh day of culture, cells spread all over the flask. In passage three, 4×10^4 cells were used to be seeded on *Cerastoderma* shell blocks. After autoclave sterilization, shell blocks were transferred into tissue culture dishes, sterilized by exposing to UV for 1 hour and washed with

PBS. Following an overnight incubation with 1mm DMEM, 10% FBS and antibiotics, shells were used for WJ-MSCs seeding (3, 10).

5. Cell viability assay and SEM

Proliferation of cells attached and grown in *Cerastoderma* shell blocks was measured using 3-(4,5- dimethyl thiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay. 12 h after cell seeding, 20 µl of MTT (5 mg/ml) was added to each well and incubated for 4h at 37°C, then 150 µl of DMSO was added to solubilize the crystals for 20 min at room temperature. The optical density was determined with a spectrophotometer (MultiskanMK3, USA). Absorbance was measured at 490 nm using an ELISA reader (Spectra MAXM3, USA) (18).

To study the structure of *Cerastoderma* shell and behavior of cells while culturing on it, SEM was performed 3 days after cell seeding. The *Cerastoderma* shell blocks, seeded with cells, were washed with PBS buffer, and fixed with 2.5% paraformaldehyde solution at 4^0C for 16h. The samples were post fixed with 0.1% osmium tetroxide solution and dehydrated in a series of ethanol (25%, 50%, 75%,95%,100%, and100%). The dehydrated samples were sputter coated with gold, and viewed using SEM (JOM-6360; JEOL, Tokyo, Japan) (19).

6. Osteoblast induction

To induce osteoblastic differentiation, cells culture medium was replaced with a media supplemented with an osteogenic stimulant (OS), that is, 10mM b-glycerophosphate (Sigma, USA) 50 µg/mL ascorbic acid (Sigma, USA), and 0.1 mM Dexamethasone (Sigma, USA). The OS-containing media was renewed every 2 days during osteogenic induction (20).

7. ALP activity assay

The degree of osteoblastic differentiation of cells was evaluated by determining ALP activity. 5 days after treatment with the osteogenic induction medium, the adherent cells were removed from the shell blocks, homogenized in PBS with 1% TritonX-100. A 100-µL aliquot of the suspension was then mixed with 200 µl of 0.1M glycine NaOH Buffer (pH10.4) and 100 µl of 15mM p-nitrophenyl phosphate (p-NPP) (Sigma, USA) and incubated for 30 min at 37^0C. The reaction was terminated by adding 0.1N NaOH. The Degree of p-NPP hydrolysis were determined using an ELISA reader (SpectraMAXM3, USA) at 410nm; p-nitrophenol (p-NP, Sigma, USA) was used as a standard. Protein concentrations were measured using a BCA protein assay reagent kit (Pierce, Rockford IL, USA) according to the manufacturer's instruction (21).

8. Preparation of rice bran extract (RBE)

Brownish rice bran was purchased from a rice supplier in Sari, Iran. In order to remove dust, it was washed and dried. Extraction was carried out by alcohol 70% using maceration method, in which alcohol was added 10 times of the substance weight, followed by shaking at room temperature for 48h. Finally, the supernatant was filtered via filter paper and condensed to dryness at room temperature using a rotary evaporator (14).

9. Proliferation and osteogenic differentiation assessment of cells in presence of RBE

To investigate the cytotoxic and proliferation effect of rice bran on WJ-MSCs, 5×10^4 cells were cultured on 24-well plates and treated for 72 h with various concentrations (20–150 µg/ml) of rice bran. Then, 100µl of MTT solution (5mg/ml in PBS) was added to each well and incubated at 37°C for 4 h in darkness. Subsequently, the medium was replaced with 1ml DMSO and the plate was kept at room temperature for 20 minutes. The absorbance value was measured at 570nm and the percentage of viable cells were calculated via following formula: Viability= mean absorbance of sample /mean absorbance *100 (18).

To study the effect of rice bran on differentiation, 5×10^4 cells were cultured into 24-well plates. At 80% cell confluency, the medium was replaced with osteoblastic differentiation medium including 10% FBS DMEM, 50 µg / ml ascorbic acid, 10 nm Mg dexamethasone and 10 mM beta-glycerol triphosphate. Then, cells were treated with a concentration of 40 µg/ml of RBE (Test group) or 1% DMSO (Sham) for 14 days (14). Cells were stained with Alizarin Red to confirm the presence of calcium deposits (19).

10. Statistical analysis

Statistical analysis was performed using SPSS 16.0 software by running a one-way ANOVA. $P < 0.05$ was also considered to indicate statistical significance.

Results

1. Characterization of shells by X-ray diffraction

The results of the X-ray diffraction are shown in Figure 1. The aragonite mineralogy was verified for the whole shell and we recorded no trace of calcite or other minerals.

2. Characterization of WJ-MSCs

Oct-4 is a magic stemness marker which is highly expressed in embryonic stem cells. Our data confirmed the expression of Oct-4 in WJ-MSCs (Figure 2).

3. Cell viability assay and SEM

MTT results showed that only one day after cell seeding, about 80 percent of cells attached to the ventral side of the shell. There was no significant difference between cells cultured on plastic plates and cells cultured on shells while there was a significant difference compared with cell-free shells (Control) at 420 nm (Chart 1).

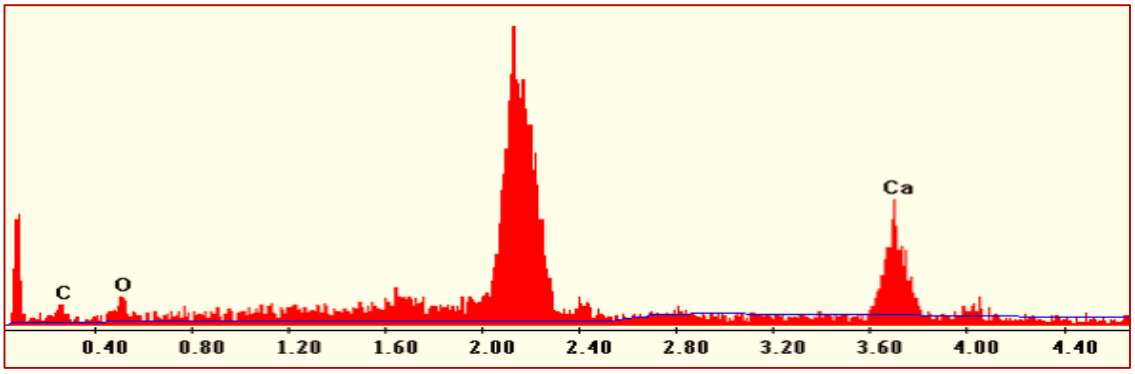

Figure 1. XRD of the ventral side of shells. Aragonite composition of *Cerastoderma* shell was assessed. The three peaks shown in the graph are related to the most abundant elements in the shell, including calcium, oxygen, and carbon, which together form calcium carbonate, the greatest composition in shell structure.

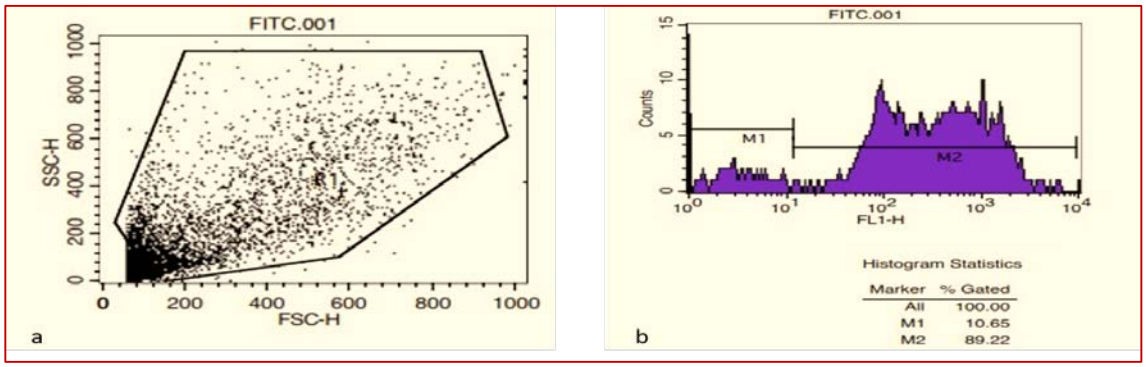

Figure 2. Oct-4 stemness marker detection by flow cytometry. WJ-MSCs expressed high levels of Oct-4 (a). As it is known in histogram(b), 89% of cells expressed this marker (cells in M2 area).

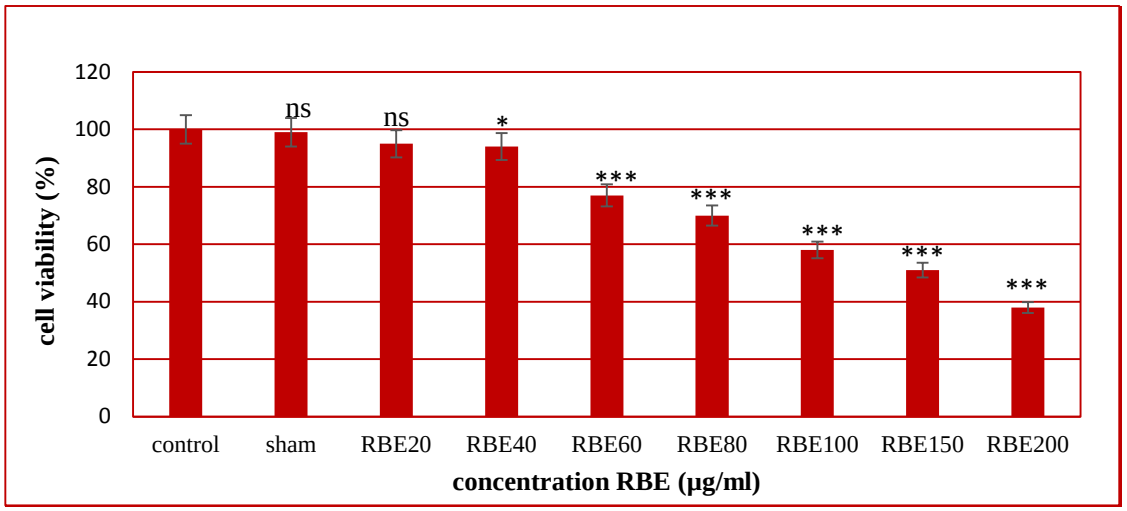

Chart1. Surviaval rate of seeded cells on shell blocks of *Cerastoderma*. Attachment and proliferation of cells onto the scaffold were proved using MTT assay, about 85% of seeded cells adhered and proliferated on shell (mean ± SD, $^{***}P < 0.001$).

We used SEM to observe the structure of the ventral side of shell blocks. The results of SEM analysis showed that the ventral side of the shell had an irregular surface and embossed nonporous morphology (Figure 3a).

Cellular morphology and interaction of cells seeded on shell blocks were visualized using SEM after 3 days of culture. On the shell scaffold, cells grew on the surface and maintained their fibroblast-like shape. Furthermore, they formed bridges between the surface and other spaces (Figure 3b).

4. ALP activity assay

To determine whether WJ-MSCs differentiated into osteoblasts during OS treatment and while culturing on natural *Cerastoderma* shell, ALP activity was measured. ALP values were normalized for total protein content and expressed as µmol/min/gr percent protein. When treated with OS, ALP activity increased in WJ-MSCs, similar to that of cells cultured in plastic dishes (Chart 2).

5. Proliferation and osteogenic differentiation assessment of cells in presence of RBE

Our findings showed that survival rate of Wharton's Jelly derived-mesenchymal stem cells while treating with rice bran extract decreased in a dose-dependent manner. We found that doses below 60 µg / ml had a small cytotoxic effect on mesenchymal stem cells. As shown in Figure 6,

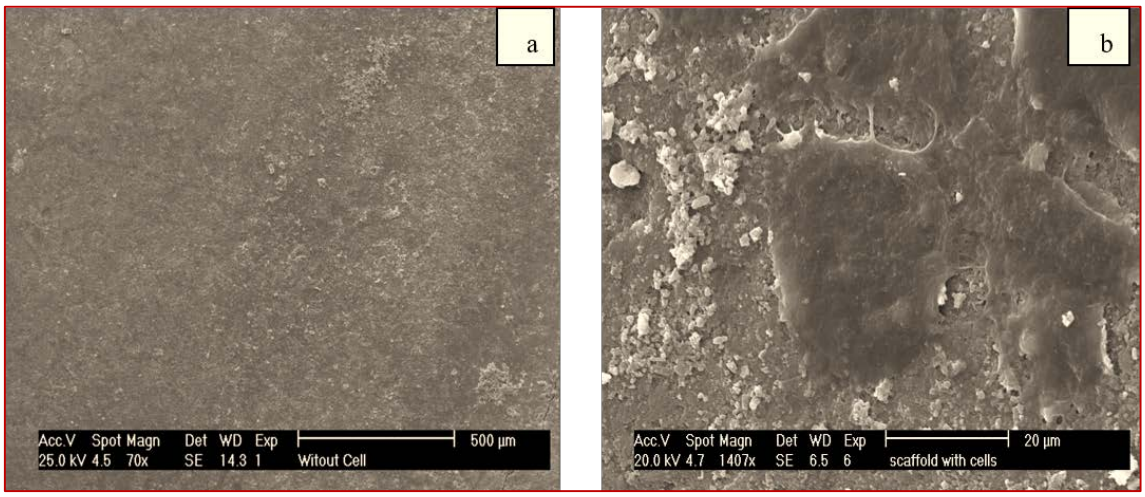

Figure 3. SEM micrographs of WJ-MSCs and the shell blocks. Acellular, irregular and nonporous morphology of ventral side of shell (a), WJ-MSCs on the ventral surface of *Cerastoderma lamarcki* (b).

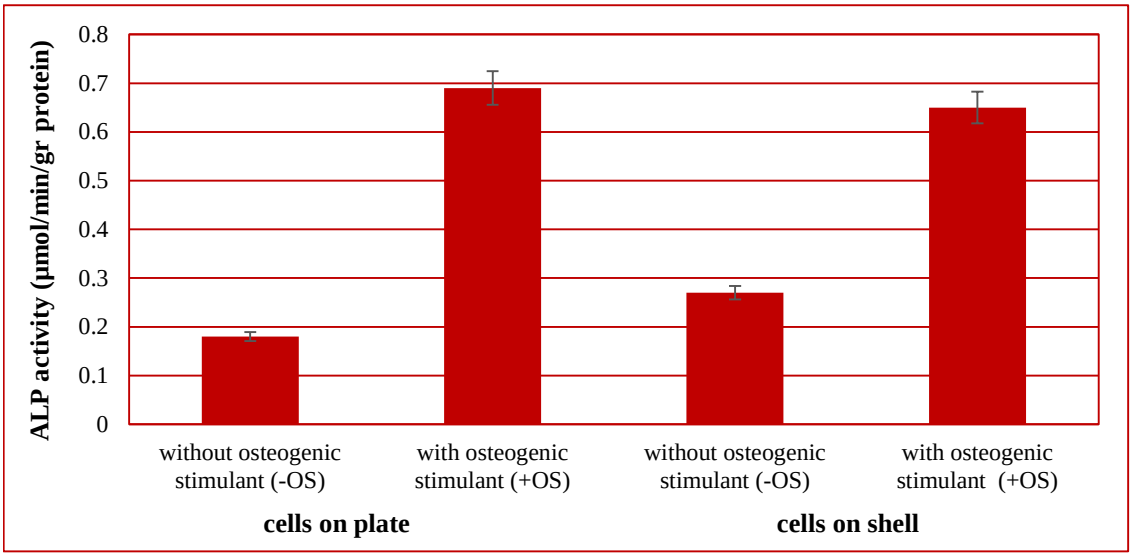

Chart 2. Alkaline phosphatase (ALP) activity of Wharton's Jelly-derived MSCs cultured on natural *Cerastoderma* shell. Cells were cultured in the presence/ absence of an osteogenic stimulant (OS). ALP activity significantly increased in Wharton's Jelly-derived MSCs cultured on shells in the presence of OS (mean + SD, *** P< 0.001).

while the survival rate of cells treated with 20 µg / ml of extract is not significant compared to control group, cells treated with the concentration of 40 µg / ml showed a survival percentage of 90.3%. Thus, this dose had a minimal cytotoxic effect and can be used for osteogenic differentiation (Chart 3).

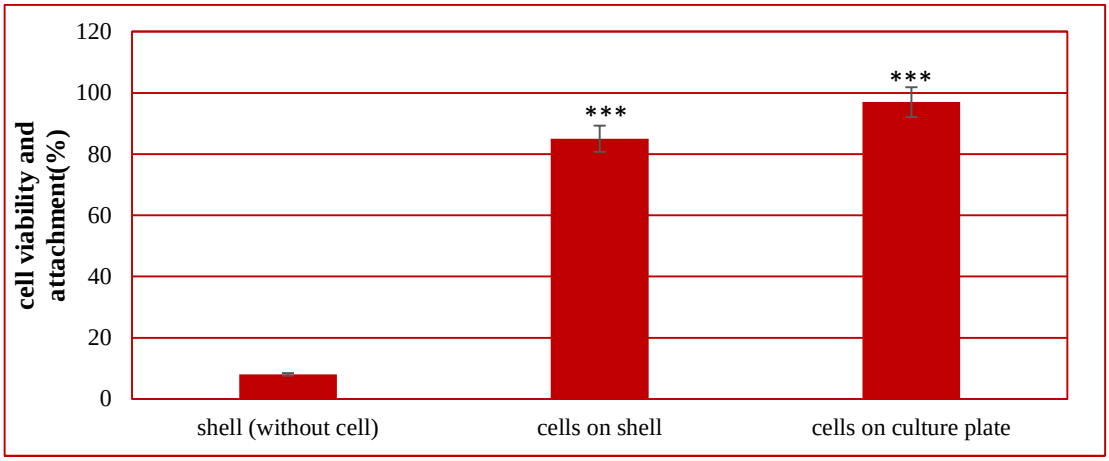

Chart3. Effect of RBE on the survival rate of WJ-MSCs. Cells were treated with RBE and 1% DMSO (sham group) for 72 h. There is no significant difference between sham and control groups (*** P <0.001, * P <0.05, ns P> 0.05%; mean ± SD).

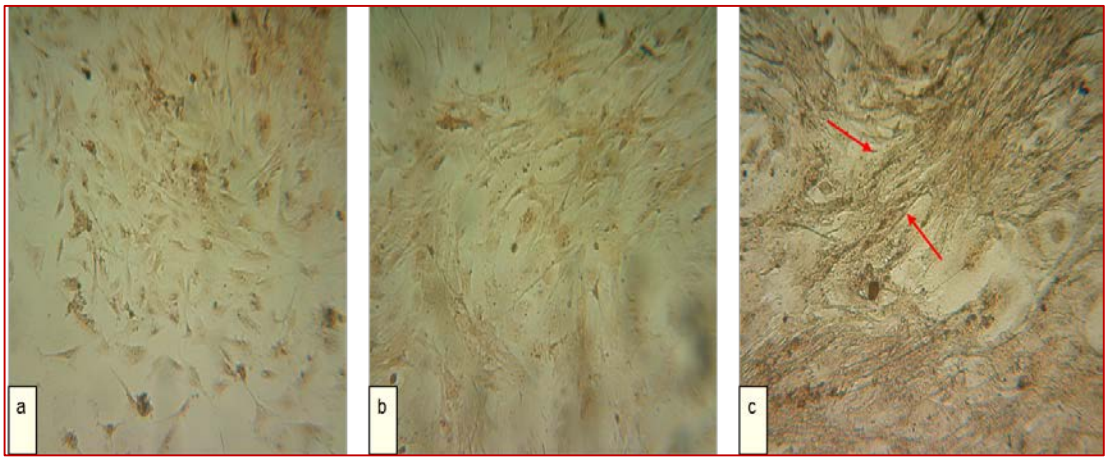

Figure 4. Alizarin red staining of Wharton's Jelly-derived MSCs treated with RBE after 14 days. Control group, non- treated cells (a), sham group, treated with 1% DMSO concentration (b), cells treated with 40 µg / ml of RBE (c) (arrows show the reddish- brown calcium deposits).

Alizarin Red staining showed that cells treated with 40 µg / ml of rice bran extract display reddish- brown calcium deposits. While undifferentiated mesenchymal stem cells lack extracellular calcium deposits, they can appear extensively in differentiated osteoblasts both *in vitro* and *in vivo*. In fact, calcium deposits indicate the successful differentiation of mesenchymal stem cells into osteoblasts (Figure 4).

Discussion

It is generally accepted that marine organism especially marine sponges provide a wide range of applications in biomedicine due to their antitumor, antiviral, anti-inflammatory and antibiotic properties. In fact, structural characteristics and mineral content of marine sponge have made this organism an ideal candidate for bone tissue engineering. On the whole, the rich biodiversity of marine life presents many other organisms which can also be considered as unique and effective bone scaffold (22). In this study, we investigated the biomedical applications of a local bivalve in Caspian Sea, Iran. Unlike other groups of mollusks, there is no previous study about the use of this bivalve in bone tissue engineering

applications. However, its architecture and organic components could generate a considerable interest (23). The natural *Cerastoderma* shell is composed of calcium carbonate and contains small amounts of heavy metals and other organic components (24). Thus, we hypothesized that natural *Cerastoderma* shell prepared with minimal processing could provide an appropriate environment, for attachment proliferation, and differentiation of WJ-MSCs into osteoblasts. One of the most-studied mollusks is cuttlefish bone(25). Cuttlefish bone (*Sepia officinalis L.*) has two structural parts (dorsal shield and lamellae), a thick external wall and an internal matrix. The dorsal shield is a hard cover that overlays the lamellar matrix. This layer has a nonporous structure. The lamellae are separated by pillars and form chambers which are areas with spaces between 200 and 600 µm (26). The surface morphology of *Sepia* bone and its two structural parts have been studied previously. It is also shown that natural cuttlefish bone supports the adhesion, proliferation, and differentiation of MSCs. MSCs grew only on the outer surface of dorsal shield blocks, whereas they penetrated deeper into the lamellar blocks (27). In this study, we used the ventral surface of shells that have pores with the size of 1-20 µm. Thanks to these pores, WJ-MSCs adhered and proliferated on the outer surface of the shell and only few cells could penetrate into fracture parts of the shell and extend. Thus, this surface of the shell is comparable with dorsal shield of cuttlefish bone (28). Cell culture is a starting point to establish whether materials are cytotoxic or not, and the tetrazolium-based colorimetric MTT assay is a quantitative method for assessing the biological response of cells to a material, whereas the direct contact test permits qualitative assessment of the cell response after culture (29). According to MTT assay, most cells appeared healthy and viable. Thus, we found that *Cerastoderma* shell had no cytotoxic effect on WJ-MSCs. SEM study of WJ-MSCs seeded on *Cerastoderma* shell confirmed the attachment of cells to the surface of *Cerastoderma* shell. It suggests that the natural *Cerastoderma* shell allows WJ-MSCs attachment and growth. Moreover, WJ-MSCs maintained their typical fibroblast-like shape and it shows that *Cerastoderma* shell serves as a supportive material for cell growth. ALP is one of the most commonly-used markers of osteogenesis and reflects the proportion of osteogenic differentiation (30). In our study, ALP levels increased during WJ-MSCs culture on *Cerastoderma* shell. These findings suggest that natural *Cerastoderma* shell is an effective and useful scaffold for osteoblastic differentiation. According to another study, Chalisserry et.al used from nano-crystalline hydroxyapatite from parrot fish (*Scarus collana),* their MTT results showed appropriate viability of rabbit bone marrow mesenchymal stem cells in presence of hydroxyapatite isolated from fish bone. They also used from ALP and Alizarin Red for determination of osteoblastic differentiation, their study showed significant differentiation of cells with treatment by fish bon-derived hydroxyapatite. Their findings are comparable with ours and their results demonstrate our findings about usefulness of marine environment materials in bone regeneration, although one of advantages of our study is that the hydrothermal procedure of isolation of hydroxyapatite is not present in our study and our results are gained from pure natural biomaterial without any extra Processing(31). In the present study, we used the ventral side of *Cerastoderma lamarcki* shell and we believe that doing studies on the dorsal side of this bivalve and comparing the results could be even more interesting. Using this scaffold as an implant in *in vivo* models and using human stem cells and checking the biocompatibility of the scaffold would also be promising.

Our second natural material – rice bran extract– also showed some interesting and effective results while co-culturing with mesenchymal stem cells.

Several studies have aimed to identify natural products or healthy foods that prevent or reduce osteoporosis with the least side effects. Rice bran extract with its anti-osteoclastogenic effect is one of the most important natural products which contains various useful ingredients (20). Previous studies suggested that butanol fraction of RBE restricts the activity of MAP-kinase pathway and c-Fos /NFATc1 expression which are essential in osteoclastogenic activity (15). It is also shown that p-hydroxycinnamic acid, a photochemical agent in rice bran, inhibits the formation of osteoclast cells in mouse bone marrow (32). In another study, researchers showed that the alpha-tocotrienol in rice bran inhibits osteoclastic bone resorption by inhibiting the expression of NF-κb ligand. As mentioned above, most studies on the effect of rice bran extract have been conducted on

inhibition of osteoclasts (33). In this study, we examined the effect of rice bran extract on osteoblasts that has not yet been dealt with adequately. Osteoblasts are specialized mesenchymal cells which play an important role in bone formation and osteoclasts regulation. As differentiated, the cells acquire the ability to secrete bone matrix (34). Considering the positive effect of RBE on the control of osteoclasts differentiation, we decided to investigate the effect of this natural substance on osteoblasts differentiation. One of the most significant changes in osteoblasts differentiated cells is calcium deposition which was found in our treated cells with RBE. In this study, we used aqueous-alcoholic extract of rice bran (30:70 v/v) and investigated the effect of this alcoholic extract on both survival rate and osteoblasts differentiation of WJ-MSCs. Moon et al. obtained three other extracts of rice bran as follows: butanol, ethyl acetate and hexane. They found that only the 50 µg / ml concentration of butanol RBE had osteoclast inhibitory effects (15). In this study, 40 µg / ml alcoholic concentration of RBE, which according to our findings has small cytotoxic effects, was used to measure osteogenic differentiation.

Conclusion

To conclude, we found that *Cerastoderma lamarcki* shell as a marine bivalve and Rice Bran as an agricultural by-product both can be used as promising biomaterials in bone regeneration. We also suggest doing additional research to reveal more precise and exact details about these biomaterials.

Acknowledgments

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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