RESEARCH PAPER

Preparation of demineralized bone matrix nanoparticles as new drug delivery system and evaluation their toxicity on chicken embryo and Wharton's jelly mesenchymal stem cells

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ABSTRACT

Objective(s): Demineralized bone matrix (DBM) is an allograft bone composed of native insoluble bone morphogenetic proteins and plays important roles in skeletal development, osteogenesis, and differentiation of mesenchymal stem cells. The osteoinductive capabilities of Allogenic DBM make it a potential drug delivery system for preventive treatment in various anatomical sites. In this study, the cytotoxic and teratogenic effects of DBM nanoparticles, on Wharton's jelly mesenchymal stem cells and chicken embryos were evaluated.

Materials and Methods: DBM nanoparticles were injected into fertile eggs at doses of 10, 20, 40, 80, and 100 µM / egg. Then morphological, histological, and skeletal malformations were evaluated. Cytotoxic effects of DBM nanoparticles on Wharton's jelly mesenchymal stem cells were also assessed using MTT test.

Results: Results showed that the fetal growth abnormality occurred only in embryos treated at the highest dose tested (i.e., 100 µM / egg) and MTT test showed no cytotoxicity in low concentration.

Conclusion: These results indicated that nanoparticles do not have significant toxic effects on chick embryos and cultured stem cells. Only high doses of DBM nanoparticles reduce growth in embryos and cultured cells.

Keywords: *Chick embryo, Mesenchymal stem cells, Nanoparticles, Toxicity*

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INTRODUCTION

Nanoparticles have attracted much interest recently because of their potential use in various industries, especially the pharmaceutical industry. Nanoparticles can be categorized according to size, shape, origin, surface charge, and aggregation [1]. Despite enthusiasm for nanoparticle research, the particles can harm living organisms. As nanoparticles become more minor, they can enter into cells more efficiently, becoming more chemically active and toxic [2]. Haase et al. observed that as silver nanoparticles became smaller, they caused more cytotoxicity and cell death in THP-1 cell lines [3].

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Cationic surface charge nanoparticles interact with cell membrane and genome more easily than neutral or anionic surface charge nanoparticles. Spherical nanoparticles are less toxic and have easier endocytosis than other shapes [4]. According to a different study, proteins' secondary structure is changed when magnetic iron oxide nanoparticles (IONs) interact with egg albumen. Furthermore, different ION concentrations have different effects on chick embryos. Higher doses result in increased embryonic mortality and neuronal loss, as well as decreased embryo weight and size [5]. Pietroiusti and his colleagues showed that nano-alumina at doses of (1 µM to 16 mM) have toxicity in human brain microvascular endothelial cells (HBMECs). Nano-alumina particles increased cellular oxidative stress, altered mitochondrial functions, and decreased HBMECs viability [4, 6].

Demineralized bone matrix (DBM) is an allograft bone, which is composed of natural, insoluble bone morphogenetic proteins like platelet-derived growth factor, transforming growth factor, acidic fibroblast growth factor, and bone sialoprotein. These proteins have an essential role in skeletal development, osteogenesis, differentiation of mesenchymal stem cells, and drug delivery for prophylactic use in various different anatomical sites [7, 8]. For instance, prior research has demonstrated that DBM can stimulate *in vitro* development of MSCs into chondrocytes [9]. DBM is most commonly used in craniofacial, axial, and appendicular bone regenerative applications. Besides, it is used in bone fractures, bone cysts, bone tumor surgery, osteonecrotic lesions of the femoral head, and ankle or foot fusions [10].

Stem cells, which have been widely used in clinical studies, are unspecialized cells with the ability to self-renew, proliferate indefinitely, and generate a variety of differentiated cells. Adult stem cells are multipotent but have limited differentiation potential. They are divided into multiple types, such as mesenchymal, endothelial, hematopoietic, and other types [11]. Bone marrow, adipose tissue, umbilical cord blood, Wharton's jelly, placenta, and other bodily tissues are among the sources of mesenchymal stem cells (MSCs). The low immunogenicity of MSCs protects them against intense immune responses. Consequently, in the case of allograft transplantation of MSCs, immunosuppression is not necessary [12, 13].

We explore here the toxicity of DBM nanoparticles on two substrates. The first is the chick embryos. These embryos are classic laboratory models used in diverse research fields, such as genetics, immunology, virology, cellular biology, pharmacology, toxicology, and biochemistry [14]. Chick and human embryos have numerous similarities at the cellular, molecular, and anatomical levels. The benefits of using chick embryo as a research model include rapid development, reasonable price, accessibility, large size, and the possibility of implementing various techniques and manipulations, such as allograft and autograft transplantation, implantation of particles coated with growth factors or small molecules, and cell culture [15, 16]. Our other substrate is Wharton's jelly mesenchymal stem cells (WJ-MSCs). Because of their low cost, access to umbilical cord tissue, high reproductive potential, and low susceptibility to viral infection, WJ-MSCs are a convenient source of stem cells [17]. In this study, the cytotoxic and teratogenic effects of DBM nanoparticles, as an ideal drug delivery system, on Wharton's jelly mesenchymal stem cells and chicken embryos were evaluated.

MATERIALS AND METHODS

Preparation of DBM nanoparticles

To synthesize DBM nanoparticles, fresh cow femurs were cut into 1×1 cm segments and washed in phosphate-buffered saline (PBS). For physical decellularization, the segments were sequentially immersed in liquid nitrogen for 2 minutes and PBS for 10 minutes. For subsequent chemical decellularization, segments were submerged in a 2.5% sodium dodecyl sulfate (SDS) solution for 8h with mechanical stimulation. The segments were then sterilized with 70% ethanol. Demineralization was conducted at 37 °C with 1N HCL for 30h. The soft bone was then cut with a scalpel and converted into nano-powder using a Baloot Mill (NARYA-BM25, Iran) [18, 19].

Characterization of DBM nanoparticles

The size, shape, and surface attributes of the DBM nanoparticles were characterized with atomic force microscopy (AFM) (JPK, Germany). Additionally, field emission scanning electron microscopy (FESEM) (Supra 35VP-24-58, Germany) was used to assess the distinctiveness of the morphology and ultra-structural nature of DBM nanoparticles.

Treatment of chicken embryos

Ross-308 fertilized eggs were acquired from Arta chicken company Ardabil province Iran and maintained at 10-12 °C. To assess the biological function of DBM nanoparticles, they were dissolved in Dimethyl Sulfoxide (DMSO), and on the third day of incubation, 100 µl of the DBM solution was injected into the air sac of each egg using a Hamilton syringe at doses of 10, 20, 40, 80 and 100 µM / egg. As control eggs, eggs were injected with 100 µl of DMSO alone. The injection sites were immediately covered with melted paraffin, and the eggs were incubated at 65% humidity and 37.7-38 °C. To assess the growth of the embryos during incubation, candling was done every day. Three repeats of each assay were done for all concentrations (n=10).

Toxicity and teratogenicity evaluation

On the 19th day of incubation (HH stage 45),

treated and control eggs were opened and the embryos were weighed. The rate of mortality was calculated with the following formula.

All embryos were photographed with a digital camera (Coolpix 950) and any morphological abnormalities were evaluated. In order to evaluate skeletal defects, after skin evisceration, the embryos were submerged in a 2% potassium hydroxide (KOH) solution for three days. Embryos were then stained for three days with 0.1% alizarin red in a 1% KOH solution. For final sterilization, the embryos were placed in 100% glycerol [20].

Histopathological evaluation

For histopathological evaluation, liver tissue of treated and control embryos was collected on the 19th day of incubation and fixed in 10% formalin. Samples were dehydrated in an alcohol series and paraffin-embedded. Tissues blocks were sectioned at 5-6 µm using a microtome and Hematoxylin and eosin was used for staining. An optical microscope was used to scan the slides [21, 22].

Isolation, culture, and identification of WJ-MSC

Human umbilical cords were obtained following Caesarean deliveries with the permission of mothers at the obstetrical department of Arta Hospital, Ardabil University of Medical Sciences, Ardabil, Iran. All research procedures were carried out in compliance with the Declaration of Helsinki and the guidelines of the Ethics Committee of the Mohaghegh Ardabili University of Ardabil.

Briefly, the umbilical cords were first washed with 70% ethanol, then split into 2 cm segments and maintained in Hanks balanced salt solution. After the blood vessels were removed, Wharton's jelly was cut into 0.5 mm sections. Subsequently, the sections were cultivated in Dulbecco's modified low glucose Eagle medium (DMEM, Gibco, Germany) consisting 20% fetal bovine serum (FBS, Gibco, Germany) and 100 IU/ml penicillin/100 mg/ml streptomycin (Gibco, Germany) in 75 cm^2 flasks (SPL, Korea). The flasks were kept at 37 °C with 5% CO2 and the medium was changed every 3 days [23].

WJ-MSCs, at passage 4, were grown in adipogenesis and osteogenesis differentiation medium (Invitrogen, USA) following the manufacturer's instructions to assess multipotency. The WJ-MSCs were kept at 37 °C with 5% CO2 for 3 weeks and the medium was changed every 3 days. After 3 weeks, the WJ-MSCs were stained with Alizarin Red and Oil Red O for osteogenesis and adipogenesis. The expression of the CD29, CD90, CD105, CD34, and CD45 surface markers (BD Biosciences, USA) was verified using flow cytometric analysis in accordance with the manufacturer's instructions. Cells labeled with FITC-conjugated IgG1k, IgA, and IgM antibodies served as isotype controls. Flow cytometry was carried out using a BD FACSCalibur flow cytometer (BD Biosciences), and FlowJo v 10 (TreeStar) software was used for analysis.

Evaluation of DBM nanoparticle cytotoxicity

The MTT assay for the metabolic activity of cells was applied to determine the cytotoxicity of DBM nanoparticles in chick embryos and WJ-MSCs were exposed to DBM nanoparticles at concentrations 5, 10, 15, 20, and 25 µM. The 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), a yellow water-soluble component, is reduced by mitochondrial reductase enzyme from metabolically active cells to an insoluble purple formazan, which is the basis for the MTT assay. Seeded WJ-MSCs were positioned in microplates with wells filled with Dulbecco's modified Eagle's medium (DMEM) and incubated at 37 °C with 5% CO2. Cell viability was then examined by MTT assay after 48 h. Each well received 20 µl of MTT, which was then incubated for 4 hours without light. The medium was withdrawn after 4 hours of incubation, and formazan crystals were dissolved in 200 µl DMSO and incubated for 10 min. Using an Elisa reader, the samples' absorbance was determined at a wavelength of 570 nm [24, 25].

Statistical analysis

SPSS Statistics 20 was used for the statistical analysis. One-way analysis of variance (ANOVA) and the Tukey *Post hoc* test were used to compare statistical differences between groups. The probit test was employed to evaluate LD50. The mean and standard error of the mean were used to express all data (S.E.M). A value of $p<0.05$ was considered statistically significant.

RESULTS

Characterization of Nano-DBM

The size distribution and morphology of the particles were investigated using FESEM. The DBM nanoparticles are approximately equal in size, almost spherical, and approximately 40-50 nm in diameter (Fig. 1A). AFM findings further confirmed

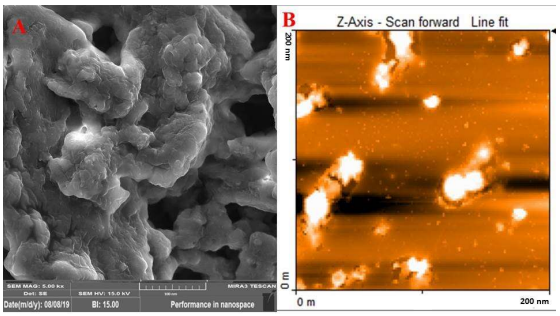

Fig. 1. A FESEM photograph of DBM nanoparticle. Photograph shows that DBM nanoparticles are approximately spherical in shape, and approximately 40-50 nm in diameter (A). An AFM image of the DBM nanoparticle surface (B)

that the nanoparticles' surface is round and the particles are spherical form (Fig. 1B).

WJSCs morphology and characterization

Medium-cultivated WJSCs display a spindle-like shape under inverted phase microscopy (Fig. 2A). Also, to verify characterization of WJSCs, surface marker expression was analyzed with FACS. The results, shown in Fig. 2B, indicated that WJSCs express CD29, CD90, and CD105. However, the hematopoietic lineage markers CD34 and CD45 are not expressed in these cells. In addition, differentiation tests were used by seeding WJSCs in osteogenic and adipogenic media to determine the multi-differentiation potential of WJSCs. After inducing cell differentiation in osteogenic media, calcium mineralization was observed using Alizarin Red S staining (Fig. 2C). Adipogenic differentiation was documented with Oil Red O staining. These developmental changes included the accumulation of intracellular lipid vacuoles (Fig. 2D).

Embryo toxicity and teratogenicity evaluation

The embryo toxicity tests indicated that

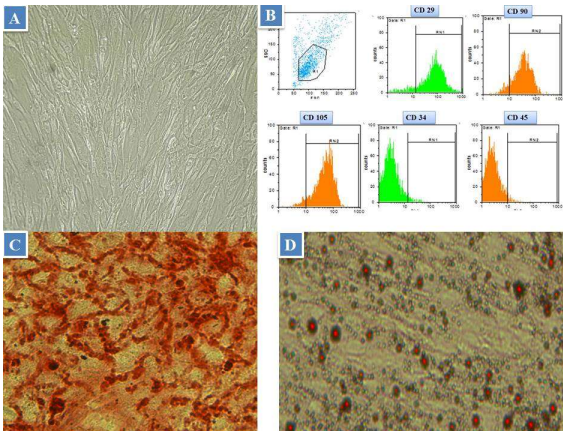

Fig. 2. (A) Human Wharton's jelly stem cells morphological characterization at passage 3 (40×). (B) Analysis of Wharton's jelly stem cells surface-marker expression by Flow cytometry at passage 3. (C) Osteogenic differentiation of Wharton's jelly stem cells after 21 d using Alizarin Red staining. Alizarin red staining reveals the presence of calcium crystals in cultured Wharton's jelly stem cells. (D) Three weeks later, adipogenic differentiation of Wharton's jelly stem cells. Differentiated Wharton's jelly stem cells have lipid droplets that appear red by oil-red-O staining

the eggs treated with DBM nanoparticles at concentrations 5, 10, 20, 40, 80, and 100 µM / egg had 91%, 88%, 83%, 74%, 62%, and 49% embryonic viability, respectively (Fig. 3). The linear relationship between the groups treated with DBM nanoparticles and the survival rate of embryos showed that LD50 (lethal dose of 50%) was 96.67 µM. Also, the embryonic weight data revealed a non-significant difference between the control group and the groups treated with DBM nanoparticles. Morphological and skeletal analyses of treated embryos only showed abnormalities of fetal growth retardation at the 100 µM dose. Also, no skeletal abnormalities were observed (Fig. 4). No abnormalities were observed according to the

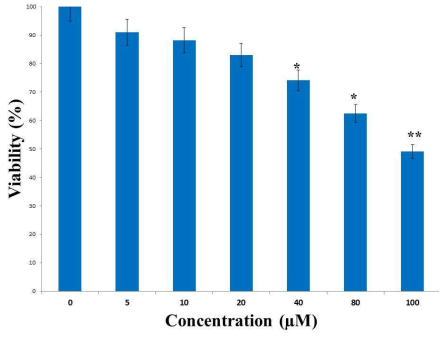

Fig. 3. Effects of *in ovo* injection of DBM nanoparticles. Figure shows the effects of different concentrations of DBM nanoparticles on chicken embryo development

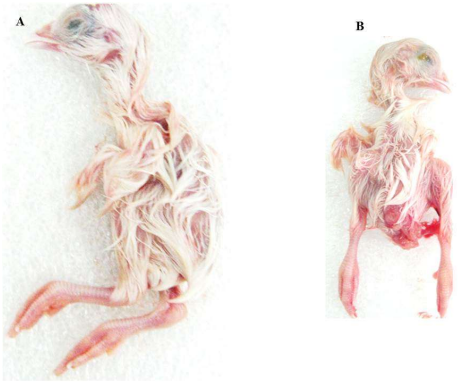

Fig. 4. Figure shows the fetal growth retardation at the 100 µM dose in treated embryos (B) compared to control group (A)

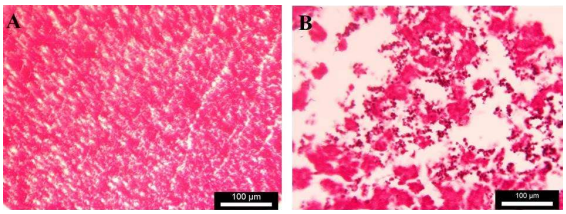

Fig. 5. Histopathological evaluation of apoptosis in liver tissue. (A) Control group. (B) Granular masses show the apoptosis formation in hepatocytes from the embryos exposed to DBM nanoparticles at the 100 µM dose

Hamburger and Hamilton scores in control group embryos [26].

Histopathology of embryos

The results of liver weight measurements showed that none of the DBM nanoparticle doses significantly suppressed organ weight. However, tissue sections from the livers revealed apoptosis in hepatocytes from the embryos exposed to DBM nanoparticles at the 100 µM dose (Fig. 5).

DBM nanoparticle cytotoxicity

To assess the toxicity of DBM nanoparticles on WJSCs, cells were incubated with DBM nanoparticles at concentrations of 5, 10, 15, 20 and 25 µM for 48 h. MTT assays showed that cytotoxicity of DBM nanoparticles, directly related to the nanoparticle concentration. There was no cytotoxicity at the lowest concentration, but as the nanoparticle concentration was increased, cell viability decreased (Fig. 6).

DISCUSSION

In recent years, significant progress has seen in nanotechnology research and applications. Nanoparticles are now widely used in drug delivery, diagnosis, and disease treatment [27]. The safety of nanoparticles, on the other hand, has not been thoroughly evaluated. Toxicity tests revealed that treating eggs with DBM nanoparticles at concentrations less than 100 µg/egg did not result in abnormalities, though growth retardation was observed at that dose. In line with this result, Abdolmaleki and his colleagues showed that treatment with salen caused fetal growth retardation in chick embryos [14]. In this regard, previous studies revealed that some nano compounds can induce apoptosis and cleave DNA, thereby causing fetal growth retardation [28, 29]. In our study, no skeletal abnormality was found in the nanoparticle-treated groups. These results are in partial agreement with previous reports

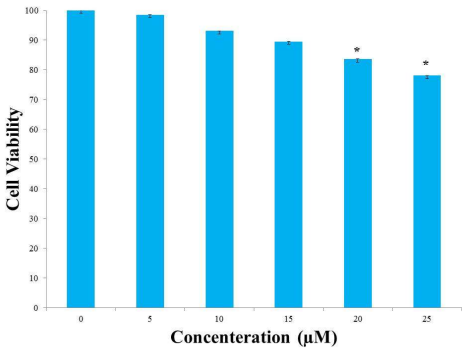

Fig. 6. Effects of DBM nanoparticles on viability and proliferation of WJSCs. The viability and proliferation of WJSCs treated with different concentrations of DBM nanoparticles showed no toxicity effect at doses below 20 µM (*P< 0.05) vs control group

that revealed low toxicity of nanoparticles in chick embryo models [5].

Our histopathological evaluation of the toxicity of DBM nanoparticles on liver tissues revealed that apoptosis in hepatocytes occurs only at a dose of 100 µM / egg. Previous studies showed that nanoparticles could induce apoptosis at high doses [30]. Khan et al. reported that human epidermal carcinoma cells treated with zinc oxide nanoparticles showed apoptosis with degradation of DNA and reactive oxygen generation [31]. Also, another study revealed that nanoparticles, which are bare and their surface is modified, can cross the blood–brain barrier and promote reactive oxygen species production [32, 33]. Recent studies showed that different types of proteins can adsorb nanoparticles, leading to interaction with cells in the body [34]. Nanoparticles can cross body barriers, such as the blood–brain barrier, which happens after toxicity [35]. Our results revealed no significant differences between the weights of vital organs of embryos treated with DBM nanoparticle up to a concentration of 100 µM. In this regard, previous studies showed that some chemical materials, like salen and some nanoparticle, like ZnS, have no negative effects on body weights of embryos [20, 32].

Cell death due to the effect of toxic compounds disrupts the pattern of cellular mitosis in existing tissue and impairs cellular contact during differentiation [14]. Results of the MTT assay showed that DBM nanoparticles showed no toxicity effect on the cell viability and proliferation at doses below 20 µM. Recent studies have also demonstrated biocompatibility and low cytotoxicity of DBM. Bo Yuan and colleagues investigated the effects of nano DBM on bone

regeneration using polycaprolactone (PCL)/-tricalcium phosphate (TCP) scaffolds. In vitro biocompatibility testing using the LIVE/DEAD and CCK-8 assays revealed that the distribution and viability of the MC3T3-E1 cell line were significantly higher in nano-DBM coated PCL/-TCP compared to PCL/-TCP scaffold. Furthermore, using nano DBM coated scaffolds in a partial long radius segmental bone defect model resulted in more bone regeneration [36]. In another study, high cell viability of L929 cells cultured in DBM/calcium sulfate composite revealed low cytotoxicity of DBM [37]. Furthermore, a group of researchers evaluated DBM/ALG biocompatibility in vitro and in vivo using sodium alginate as a DBM carrier. They tested in vitro cytotoxicity with different DBM/ALG ratios and discovered that higher DBM doses cause more cell proliferation and viability. In vivo studies revealed that implanting DBM/ALG in a 7:3 ratio in a rat model resulted in a low inflammatory response [38].

CONCLUSION

In conclusion, DBM nanoparticles have low toxic effects in chick embryos and cultured stem cells. Only in high doses (100 µM) DBM reduced the growth of chick embryos and cultured cells. Given DBM nanoparticles low toxicity, it may play a role in skeletal development and osteogenesis. Given DBM's good safety profile, further research should explore whether it can be a candidate to treat bone fractures.

ETHICAL APPROVAL

Animal protocols and surgical procedures such as animal housing and care were approved by the Laboratory Animal Center and Institutional Animal Care and Use Committee at University of Mohaghegh Ardabili (Ardabil, Iran).

Human umbilical cords were obtained following Caesarean deliveries with the permission of mothers at the obstetrical department of Arta Hospital, Ardabil University of Medical Sciences, Ardabil, Iran. All the research procedures were carried out in compliance with the Declaration of Helsinki and the guidelines of the Ethics Committee of the Mohaghegh Ardabili University of Ardabil.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

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