Osteogenic differentiation of pre-conditioned bone marrow mesenchymal stem cells with Nisin on modified poly-L-lactic-acid nanofibers

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ABSTRACT

Introduction: Human bone marrow-derived mesenchymal stem cells (hBM-MSCs) are undifferentiated cells with self-renewing ability and multi-lineage differentiation beneficial for regenerative medicine. Nano scaffolds are novel materials employed in bone repair and regeneration. Nisin is a prebiotic that can increase stem cells’ lifespan and proliferation. This study attempted to provide a proper strategy for bone marrow mesenchymal stem cells differentiation into the Osteocytes on a Poly-L-lactic-acid (PLLA) scaffold after pretreating with Nisin.

Methods: MSC osteogenic differentiation was evaluated by measuring Calcium, Alkaline phosphatase, and quantitative tests such as Real-Time PCR, Acridine Orange, Alizarin Red, Von Kossa, and others.

Results: The result of the MTT test showed that the optimal dose of Nisin prebiotic for the MSCs’ preconditioning was 200 IU/mL on the 1st, 3rd, and 5th days of culture. Real-time PCR data indicated that the expression rate of ALP, Osteonectin, Osteocalcin, and Collagen I have increased in the presence of Nisin, while the RUNX-2 gene expression has decreased. Furthermore, the results of Alizarin Red and Von Kossa tests, as well as Scanning electron microscopy (SEM), revealed that the cell proliferation in the preconditioned samples with Nisin increased significantly.

Conclusions: The study concluded that the cell proliferation and differentiation increased in samples pretreated with Nisin on the PLLA Nano scaffolds.

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characteristics make them favorable candidates for tissue engineering, while their short life span in harsh conditions is considered a limitation. Currently, preconditioning strategies have been proposed to overcome this problem in stem cell therapy. The pretreatment of MSCs with some compounds or conditions not only increases their lifespan but also sometimes improves proliferation and differentiation. The compounds used for preconditioning include some hazardous materials such as toxins, H2O2, hypoxic conditions, or many beneficial compounds such as growth factors, cytokines, and pre/probiotics [5,8–10].

2.1. Human bone marrow mesenchymal stem cell culture

2. Materials and methods

2.2. Osteogenic differentiation of hMSCs

For differentiation of MSCs, a differentiation medium has β-glycerol phosphate (1.8 g/5 mL of deionized water), dexamethasone (0.0017 g/50 mL culture medium), and ascorbic acid (0.19 g/20 mL culture medium) prepared. Following treatment with a differentiation medium, cells were incubated at 37 °C and 5% CO2 for two weeks, and every 48 h medium was changed [17].

2.3. MTT assay

Three concentrations (100, 200, and 300 IU/mL) of Nisin were evaluated for the optimal Nisin dosage used for the preconditioning of MSCs on the 1st, 3rd, and 5th days of culture. In each well of a 48-well plate 1 × 104 cells/cm2 in DMEM high glucose medium, 10% FBS, and penicillin/streptomycin in an incubator at 37 °C and 5% CO2. The cell culture medium was replaced every 48 h. The hMSCs harvested at almost 80% confluence of about 2 × 104 cells/cm2 will result in hMSCs doubling per passage. They were characterized as MSCs at passage 3 by presenting spindle-shaped cells, plastic adherence features, and possessing osteogenic differentiation abilities (the data not shown) [16].

2.4. Acidine Orange staining

Acridine Orange staining was performed to investigate the growth and proliferation of MSCs on the 1st, 3rd, and 5th days of culture. 200–300 μL of Acidine orange solution was added to each well and incubated for 5 min at 37 °C with 10% CO2. In the next step, the cells were washed twice with PBS and photographed by the inverted fluorescence microscope (Olympus-version-6.0, Carl Zeiss Lens, Germany). All statistical analyses were carried out in triplicate [18].

2.5. Alkaline phosphatase activity

The activity of alkaline phosphatase as a marker in differentiated bone marrow cells was investigated on the 7th and 14th days. At first, 200 μL of Ripa solution was added to each well and the pipette was completely taken the solution of each well with scaffolds was transferred to 1.5 mL vials and vortexed, and centrifuged (4 °C, 15,000 rpm, 15 min). Using an alkaline phosphatase kit (Pars Azmoo, Tehran, Iran) and adding 150 μL of R1 and R2 solutions, plus 50 μL of samples, which were transferred to a 96-well plate, readings at 450 nm were carried out by the ELISA reader. Values were included in the mean of the triplicate examination.

2.6. Calcium content assay

The amount of calcium sediment accumulation in differentiated MSCs to osteocytes was evaluated on days 7th and 14th of the differentiation. 200 μL of the hydrochloric acid solution was added to each well of a 6-well plate. After pipetting, the contents of each well were transferred to 1.5 mL vials and shaken for 40 min. Using a Calcium Detection Kit (Pars Azmoo, Tehran, Iran) and adding 150 μL of R1 and R2 solutions plus 50 μL of samples, they were transferred to a 96-well plate and read out at 570 nm by the ELISA reader. Three replicates were used to evaluate the average of the results [18].

2.7. Alizarin Red staining

A differentiation experiment was conducted on day 14th to evaluate the quality of calcium deposition. The wells were removed and washed once with PBS and twice with deionized water. 4% Paraformaldehyde was added to each well and placed at 4 °C for 20 min. Alizarin solution was poured onto cells and scaffolds next. Then it was washed with PBS for 5 min and photographed under an inverted microscope (OLYMPUS IX71, Olympus, Tokyo, Japan). We have made three independent replicates of this experiment [18].

2.8. Imaging by scanning electron microscopy

To investigate the morphology, and adhesion as well as biocompatibility of MSCs with PLLA scaffolds, we used scanning electron microscopy (SEM:KYKY-EM3200, Hitachi, Japan). After 14 days, MSCs were differentiated from the osteocytes all of the wells were washed with PBS. 2.5% Glutaraldehyde was added to each well and washed twice with PBS for 30 min. Then, alcohol dehydration was carried out (60–100–100%) for each well for 10 min. The fibers were coated with gold when drying and scanned by a scanning electron microscope [19].
2.9. Fabrication of poly-L-lactic acid (PLLA) nanofibers

Nanoparticles of poly-lactic acid were made using the electrospinning method. 0.43 gr of polymeric poly-lactic acid was dissolved in 5 mL of chloroform solution. Then the solution was transferred to the syringe of the device. It was subjected to a voltage of 15 kV with a speed of 0.4 mL/h. Then, the fibers were fixed on the aluminum plate [19].

2.10. Immunocytochemistry

To evaluate the Osteocalcin and Osteopontin proteins, on the 14th day of differentiation, the supernatant was removed and cells were washed with PBS. To fix the samples, 4% paraformaldehyde were added (20 min), 0.4% Triton X100 (10 min). Then, the goat’s serum of 5%, the primary antibodies of Osteocalcin (1:100), and Osteopontin (1:200) were added. After 30 min of incubation (37 °C, 5% CO2) were washed three times with Phosphate Buffered Saline (PBS). In the next step, the secondary antibody, Fluorescein isothiocyanate (FITC) (1:100), was added and incubated at room temperature for 1 h. Then, it was washed again with PBS. Cell cores were stained with a DAPI (4’,6-diamidino-2-phenylindole) color of 0.1 μg/mL and photographed with a fluorescence microscope [20].

2.11. Analysis of metastasis genes expression by quantitative RT-PCR analysis

2.11.1. RNA extraction

All tested specimens were firstly subjected to RNA isolation to determine the expression of alkaline phosphatase, Osteocalcin, Osteonectin, collagen type 1, and RUNX-2 on days 7th and 14th, all tested specimens were firstly subjected to RNA isolation. The supernatant of the wells was discarded and washed with PBS. 700 μl of RNA X plus solution was added to each well and placed in an incubator for 10 min. The plate was transferred to a microwave oven, 200 μl of cold chloroform was added to each vial, and centrifuged at 12,000 rpm for 15 min, at 7 °C. Then 100 μl of supernatant was transferred to a new vial and then added 400 μl of cold isopropanol. The supernatant was discarded again, 1 mL of 75% alcohol added to each vial, and centrifuged at 7800 rpm for 10 min at low temperature. After drying completely, 25 μl of diethylpyrrocarbonate (DEPC) Water was added to each vial and Vortexed. Nano-Drop (Wilmington, DE) measured the RNAs quality by spectrophotometric method at 280/260 nm. The absorption between 1.8 and 2.0 indicates the proper RNA concentration. We considered three replicates for each of the genes [21].

2.11.2. cDNA synthesis

500 ng of RNA were mixed with about 2 μl of each primer, 1 μl oligo-dT, and 1 μl random hexamer primer. The PCR was performed for obtained solutions at 65 °C for 5 min. Next, 2 μl including RTase Buffer 10X and O-1MDTT, 1 μl of reverse transcriptase, RNase inhibitor, and DEPC water added up to a final volume of 20 μl. The new times and temperatures were set for 5 min at 25 °C, 60 min at 55 °C, and 4 min at 80 °C for 35 cycles [21].

2.11.3. Real-time PCR

After cDNA synthesis, they were subjected to real-time PCR protocol according to the kit manufacturer’s instructions. It was performed using DNA primers and adding SYBER-green qPCR Master Mix. Then the vials were put in a Rotorgene (Corbett) real-time analyzer. The times and temperature settings included 20 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C for 38 cycles [21].

2.12. Statistical analysis

All experiments were repeated three times. The results were reported as mean ± SD. Statistical analysis was performed using SPSS software by t-test. P ≤ 0.05 is considered for significant changes. Real-time PCR data were analyzed using Rotor-Gene Q and REST software.

3. Results

3.1. Results of MTT assay

An optimal and non-lethal optimum for the MTT test was performed to determine the optimum dosage of Nisin for MSC preconditioning on days 1, 3, and 5. The optimum dosage of Nisin that survived more than 80% of cells was 200 IU/mL. It was observed that the Nisin dose of 200 IU/mL was appropriate for cell viability, and there was no significant difference between groups during 5 days (p > 0.05) (Fig. 1).

3.2. Results of Acidine Orange staining

The cell apoptosis and growth and proliferation of MSCs were performed qualitatively with other Acidine staining on days 1, 3, and 5. The MSCs proliferation on the PLLA scaffold and the Nisin preconditioning were recognized as normal compared to the control group. So, neither Nisin nor the scaffold was cytotoxic for MSCs, and none of them increased the apoptosis rate in the MSCs (Fig. 2).

3.3. Results of alkaline phosphate enzyme activity

Determination of alkaline phosphatase activity on the days 7th and 14th of osteogenic differentiation of MSCs was performed. The alkaline phosphatase enzyme activity level did not differ significantly between the PLLA and Nisin groups and control on day 7th (p > 0.05). But on day 14th, the variation of enzyme activity in the PLLA + Nisin group (P ≤ 0.01) and the Nisin group (p < 0.05) exhibited a significant increase in comparison to the control group (Fig. 3a).

3.4. Results of calcium content

Calcium sedimentation in differentiated bone marrow cells was notable on the days 7th and 14th. On day 7th, differentiation in both PLLA and Nisin groups was significantly increased compared with the control group (P ≤ 0.05), showing Calcium sedimentation. Although, MSCs exposed to both PLLA + Nisin simultaneously

![Fig. 1. Proliferation of BM-MSCs on scaffold and Nisin and TCPs during 1, 3, and 5 days’ culture period, BM-MSC: Bone marrow mesenchymal stem cell.](image-url)
displayed more increase in Calcium contents with a significance of \( P < 0.01 \) than that of the control group. On day 14th, variation of Calcium sedimentation rate in the PLLA group and the Nisin group revealed a significant increase than the control group (\( P \leq 0.01 \)). Although, the Calcium sedimentation rate in the PLLA + Nisin group was significantly higher than the control group (Fig. 3b).

3.5. Alizarin Red and Von Kossa staining

Calcium and mineral sediments were evaluated using Von Kossa coloring on the 14th day of differentiation. The calcium sedimentation rate in both scaffold and Nisin groups increased on the 14th day of differentiation compared with the control group (Fig. 4). In the calcium sedimentation study, using Alizarin Red staining, the number of minerals in both PLLA and Nisin groups was more than in the control group on the 14th day of differentiation (Fig. 4). Von Kossa and Alizarin Red staining confirmed the osteogenic differentiation in BM-MSCs in the PLLA + Nisin group.

3.6. Morphological study of the differentiated cells

The morphology, adhesion, and biocompatibility of BM-MSCs on the PLLA scaffold were investigated on day 14th of differentiation. The images by scanning electron microscope (Fig. 5) showed the BM-MSCs on the PLLA nanofibers morphology with the magnitudes of 3X and 10X. The SEM confirmed the extent of adhesion of the PLLA scaffold by the image with 1X magnification. However, the number of differentiated cells in the PLLA + Nisin group was estimated from the SEM image with 300 X magnitude.

3.7. Immunocytochemical staining of differentiated BM-MSCs

A qualitative study of Osteocalcin (Fig. 6a), and Osteopontin (Fig. 6b) proteins, was investigated on day 14th of differentiation.
Immuno-fluorescence staining was used to confirm the differentiation of BM-MSCs. The levels of Osteocalcin and Osteopontin proteins in differentiated cells in the scaffold and Nisin groups increased compared to the control group.

3.8. Real-time PCR analysis

The differentiation of BM-MSCs was done using Real-Time PCR to investigate the expression of alkaline phosphatase, Osteocalcin, Osteonectin, collagen type 1, and RUNX-2 on days 7 and 14.

On day 7th (Fig. 7a), the alkaline phosphatase gene expression, as a marker of differentiation, increased in the MSC-PLLA-Nisin and MSC-Nisin groups compared to the control group (P < 0.01). Moreover, the Osteonectin gene expression was elevated 5-times in the MSC-PLLA-Nisin group than that of the control group. The collagen type 1 gene expression was significantly higher in MSC-PLLA-Nisin and MSC-Nisin groups than in the control group (P < 0.01). On day 14th, the RUNX-2 gene expression, as the primary gene in the differentiation conditions, was significantly increased in the MSC-PLLA-Nisin group in comparison with the control group (P < 0.01) (Fig. 7b). The distinction between alkaline phosphatase, Osteonectin, Osteocalcin, and type 1 collagen in the MSC-PLLA-Nisin group was significantly higher than in the control group (P < 0.01). On the other hand, the distinct expression of the RUNX-2 gene in all groups decreased on day 7th.
4. Discussion

This study has referred to the effect of Polycarboxylic acid scaffold and probiotic Nisin on the differentiation of human bone marrow mesenchymal stem cells.

The concentrations of Nisin examined in this study were 100, 200, and 300 IU/mL. The MTT assay tested the scaffold toxicity and found an appropriate dose strengthening mesenchymal stem cells. We observed that the scaffold did not produce any toxicity on MSCs at a 200 IU/mL dosage. Thus, this did select as the appropriate dose.
of Nisin for MSC’s preconditioning. The result goes along with our previous studies [19,21,22].

According to some researchers, inspecting the chitosan scaffold by the MTT-based assay revealed that it did not effectively induce the adipocyte MSCs differentiation [2]. Checking also out the MTT test showed non-toxic effects of Polycaprolactone scaffold on hMSCs [8]. Moreover, the MTT test showed no toxicity Polycarboxylic acid scaffold for the MSCs differentiation [4]. There was no toxicity of commercial PLA and its influence on the cells’ viability and proliferation [22]. Consistent with these researches, we also observed that the scaffold did not produce any toxic agents threatening the mesenchymal stem cells.

This study using Alizarin Red staining determined that MSCs can produce calcium deposits during differentiation. The results illustrated the differentiation process by showing calcium deposits secreted from differentiated bone cells. Moreover, Von Kossa’s staining of calcium deposits in the extracellular matrix confirms that calcium sediments are completely visible after 14 days of differentiation of bone marrow MSCs in osteoblasts [23]. In 2016, Paulson and his colleagues reported that calcium deposition in stem cells provides a suitable basis for differentiating MSCs [24]. The osteoblast secret alkaline phosphatase contributes to the degradation of mineral pyrophosphate for the bone organization [1]. So, then, the study evaluated the volume of alkaline phosphatase on the days 7th and 14th. The results showed that alkaline phosphatase activity is the early indicator of osteoporosis. So, utilizing a lactic acid poly-scaffold does not inhibit the secretion of alkaline phosphatase and cell mineralization. Nisin probiotics also have a booster effect on cell differentiation. It increased alkaline phosphatase levels in differentiated cells. On the 14th day of differentiation, this effect was quite distinctive. Furthermore, the present study investigated the alkaline phosphatase activity in the bone marrow MSCs differentiation on Nano-hydroxyapatite/collagen and poly-lactic-co-glycolic acid substrate. The data showed that alkaline phosphatase activity reached its maximum in these cases. Additionally, Von Kossa and Alizarin Red staining confirmed the osteogenic differentiation in BM-MSCs in the PLLA + Nisin group more than that of the control and even more than each one alone.

However, ALP activity in MSCs and MSC-P increased from 7 days to 14 days, whereas Ca deposition did not change. There must be factors that reduce calcium deposition in the next few days. Intracellular Ca$^{2+}$ chelation and inhibition of TRPM7 can suppress mineralization. TRPM7, the seventh member of the M family of cation channel receptors, is involved in function in microcalcification. Increasing Mg$^{2+}$ also can reduce calcium deposition. Therefore, although the amount of alkaline phosphatase remains high, various factors can reduce calcium deposition [25]. In addition to markers in bone marrow cells, Osteocalcin, Osteonectin, and collagen type 1 differed significantly on days 7th and 14th. Accordingly, on day 14th, the differentiation of these markers showed a significant increase. RUNX-2 plays an essential role in osteoblasts’ development and chondrocyte maturation. RUNX-2 also increases the binding affinity to DNA and the stability of proteins. Following the previous report [9], the RUNX-2 level remains at the highest level in non-adult osteoblasts, and RUNX-2 decreases when MSCs differentiate into osteoblasts.

In further studies, the differentiation process was investigated in three groups of bone marrow, fat, and somatic bone marrow MSCs on biochemical nanocomposite scaffolds and Polycaprolactone. The obtained results indicated that the amount of calcium accumulation in differentiated bone marrow MSCs was more than that of the two others [6]. Similarly, our results also revealed that the calcium accumulation in samples isolated on nanocomposite scaffolds showed highest than that of control samples.

Regarding the possible mechanism of osteogenic differentiation by PLLA, it should be noted that: managing the cells’ mechanical, physicochemical, and biological environments is substantial for improving cellular activities, like proliferation, migration, and differentiation [29]. The Osteogenic differentiation medium contains Ascorbic acid, Dexamethasone, and β-glycerophosphate. The Osteogenic differentiation medium components and growth factors can induce cellular differentiation responses. PLLA plays a role in cell spreading, morphology, and differentiation, possibly by allowing maximum exposure of the cells to differentiation components and growth factors [30].

PLLAs serves as a mechanical substrate for cells and bioactive factors. Nanofibers incorporate into tissue engineering, can provide a suitable surface area for cell cultivation. High porosity, interconnected porous scaffold, and large surface enhance cell growth. The nanofibrous scaffold has the ability to improve cell adhesion and growth rate [31].

We employed preconditioned bone marrow MSCs with both prebiotic Nisin and PLLA scaffolds. We also examined the mRNA expression of the RUNX-2, Osteonectin, Osteocalcin, collagen, and alkaline phosphatase genes. The results presented additionally the more gene expression of the RUNX-2 on day 14th rather than on day 7th. Besides, the amount of Osteocalcin expression in this study decreased concurrently. The results were in contrast with most previous studies. They suggested that the Osteocalcin gene expression in differentiated embryonic MSCs of osteoblast was investigated on Nano-hydroxyapatite/collagen/collagen/polylactic scaffolds. It determined that the gene expression was the highest in the scaffold group than in the other groups [26–28,32].

The present study concludes that due to the human need for several days to repair bone damage, the development of effective scaffolds that can be harmless to the body is necessary. In the present study, the structure of Polycarboxylic acid scaffolds exhibited the potential to support the growth, proliferation, and differentiation of human bone marrow MSCs. Also, it has biodegradability and compatibility accompanying mesenchymal living cells. Besides, bone indices such as alkaline phosphatase activity, calcium sedimentation, and bone marrow gene expression confirmed this evidence. Nisin prebiotic conferred explicit and adequate confirmation, which improves the differentiation process of bone marrow MSCs.

5. Conclusion

The study concluded that the cell proliferation and differentiation increased in samples pretreated with Nisin on the PLLA scaffolds.

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Authors’ contributions

Sadraei, Halabian, and Khakpai reviewed the literature, outlined it, and wrote the manuscript. Chollasi and Halabian edited the manuscript and Jalali prepared figures. All authors read and approved the final manuscript.

Availability of data and materials

The data (scaled and coded) that support the findings of this study are available on request from the corresponding authors. The data are not publicly available due to privacy restrictions.
Declaration of competing interest
All authors declare that they have no conflict of interest.

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