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Effect of mesenchymal stem cells and their niche on diabetic and osteoporotic wound healing following osteogenic differentiation and bone matrix formation *in vitro*

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Abstract

Objectives: Mesenchymal stem cells (MSC) and their secreted factors (*i.e.* niche) are becoming growingly popular in bone regeneration. The mechanisms of this effect can be investigated through *in vitro* models which are cost-effective methods used for determining the effectiveness of new products in experimental and clinical applications. In the present study, we established an experimental diabetic osteoporosis model in a high-glucose culture medium with no estrogen supplement to investigate the effect of MSC and their niche which their factors secreted into 24 hours medium on osteoblastic differentiation, formation of bone islets, and the wound healing model induced by scratch assay.

Methods: A culture medium of adipose-derived rat MSC (ADMSC) with no estrogen supplement was used for cell growth to assess osteoblastic differentiation and bone islet formation. A wound model was induced using the scratch assay to investigate the effect of the model on the parameters of wound healing. Cell growth and viability was assessed using MTT assay, cell migration and differentiation and the amount of wound closure were assessed based on the expression of CD44, CD45, and CD73, and osteoblast differentiation was evaluated using Alizarin Red S and von Kossa staining. Morphological observations were performed using an inverted phase-contrast microscope and h-score was assessed with immunohistochemical staining.

Results: The use of osteogenic medium with estrogen supplement led to MSC growth and migration as well as bone islet formation. The use of a high-glucose medium without estrogen supplement inhibited MSC differentiation and bone islet formation. The administration of MSC and niche promoted the wound healing initiated by the administration of the scratch assay and this promotion was significant in terms of all the parameters of wound healing.

Conclusion: The results indicated that the therapeutic effect of MSC and niche could be used as an effective treatment model in wound healing in patients with diabetic osteoporosis. Moreover, this model could be a cost-effective method for the new treatment products to be applied in dental and orthopedic practice prior to animal experiments and clinical trials.

Keywords: diabetic osteoporosis; *in vitro*; mesenchymal stem cell; niche; osteoblastic differentiation; wound healing

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Introduction

Mesenchymal stem cells (MSC) are important cell-based therapy products due to their regenerative properties and ability to give rise to cells of various lineages. These cells have the potential to differentiate into osteoblasts and osteocytes both *in vitro* and *in vivo*, which makes them excellent candidates for bone tissue regenerative therapy.[1] MSC can be easily obtained from various sources such as adipose tissue and be prepared for use after being produced in laboratories with Good Manufacturing Practice (GMP) standards. Early-phase studies of MSC in clinical conditions including ischemia, Crohn's disease, ulcerative colitis, and liver diseases have been completed and MSC therapies have been initiated thereafter.[2] Additionally, the clinical use of MSC in numerous bone diseases has also

been initiated.[3] MSC have the potential to differentiate into osteocytes and chondrocytes both *in vitro* and *in vivo*, which makes them excellent candidates for bone and cartilage diseases in clinical trials.[1] Moreover, it has also been suggested that MSC can also be used in the presence of osteoporosis.[4–6] On the other hand, long-term exposure to the high glucose effect leads to changes in bone metabolism and microarchitecture, thereby resulting in decreased healing rates in patients with chronic and non-controlled diabetes mellitus.[7] It has also been suggested that the effect of oxidative stress, inflammation, and drug use on osteoblasts and osteoclasts produce weak bones with increased fracture risk.[8]

MSC are pluripotent cell-based therapy products that can be derived from bone marrow, adipose tissue, placenta, amniotic fluid, and fetal tissues. MSC can be easily obtained and expand for therapeutic purposes. These cells, unlike hematopoietic stem cells (HSC), can be characterized as negative for CD34 and CD45 and positive for CD29, CD44, CD71, CD90, CD73 (SH3/SH4), CD105 (SH2), CD106, and CD124.[9–12] Regenerative medicine has the potential to heal or replace damaged tissues and organs using biological products. Adipose-derived MSC (ADMSC) have recently emerged as popular therapeutic products in regenerative medicine, particularly in the treatment of inflammation and autoimmune diseases. These cells are considered to inhibit bacterial growth and colony formation in diseases such as osteomyelitis and cystic fibrosis. MSC and their secreted factors (*i.e.* niche) have been accepted as mainstay therapeutic products with no side effects in clinical practice and their distinct modifications continue to emerge every passing day.[13] They have the potential to decrease inflammation in damaged tissues, thereby promoting immunomodulation and regeneration. Moreover, MSC also accelerate regeneration and provide relatively better healing in critical volume defects.[14]

Previous study indicated that the osteoblastogenesis mineralization of rat bone marrow stromal cells is inhibited in hyperglycemic culture *via* activation of the Notch2 signaling pathway.[15] Another study evaluated diabetic rats induced with streptozotocin (STZ) and revealed that the transplantation of CXCL13-stimulated bone marrow stromal cells (BMSC) in culture increased cell proliferation rate and *in vivo* enhanced the corresponding ALP expression in bone healing.[16] Moreover, it has also been reported that high glucose levels may affect the bone cells in culture medium.[17] Other studies indicated that the administration of human diabetic serum into MSC culture medium led to reduced osteogenic differentiation in association with high glucose levels and could also be an important factor for diabetic osteoporosis.[18] A study by Qu et al.[19] examined the effect of miR-449 on osteogenic differentiation and its underlying mechanism in human bone marrow-derived mesenchymal stem cells (hBMSC) using high glucose and free fatty acids treatment (FAT) and revealed that after culturing for 14 days, the treatment dramatically decreased mineralization of hBMSC and resulted in impaired bone islets. The study also showed that the miR-449 mimics decreased the protein expression levels of runt-related transcription factor 2 (Runx2), ALP, collagen I, osteocalcin (OCN), and bone sialoprotein (BSP), which were significantly increased by miR-449 inhibitors. Another study that investigated potential abnormalities of bone marrow-derived MSC (BMMSC) in a rat menopause model established by ovariectomy revealed that proliferation, migration, and differentiation of osteoclasts was decreased, and increased by Wharton's jelly-derived MSC.[20] In an experimental study, ADMSC were cultured to mimic diabetic osteoporosis and the results indicated that the osteopontin (OPN) and Runx2 expressions of ADMSC were decreased and there was also a noticeable reduction in mineralization, which were associated with DNA methylation and Wenti signal.[21] Likewise, some other studies induced a rat diabetic osteoporosis model with STZ and high-fat diet and reported that the administration of the model with plant extracts improved diabetic osteopenia and also prevented oxidative stress and apoptosis in the fracture induced in the femur.[22,23]

Chen et al.[24] investigated the effect of Runx2 on osteoblast differentiation in high-glucose condition and reported that the expression of Runx2, ALP, OC, and OPN, as well as ALP activity and Alizarin Red S staining decreased significantly, whereas administration of 10 mM PI3K/AKT inhibitor LY294002 eliminated this favorable effect. The authors also noted that Runx2 reversed high glucose-induced inhibition of osteoblast differentiation *via* modulation of PI3K/AKT/GSK3b/b-catenin pathway. Some other studies indicated that estrogens had beneficial effects on osteoblasts during differentiation and also increased their survival and mineralization capacity.[25–28] Additionally, it has been reported that estrogen deficiency has a repressive role on MSC *via* miR-133 and that the expression of Runx2 and Osterix decreases ALP activity as well as the formation of mineralization nodules, which could be significant factors for MSC in patients with postmenopausal osteoporosis.[29]

In the present study, an experimental diabetic osteoporosis model was induced in a high-glucose culture medium with no estrogen supplement, which allowed osteoblastic differentiation of MSC and the formation of

bone matrix. By using this model, we aimed to investigate the effect of MSC and their niche (*i.e.* factors secreted into 24-h medium) on osteoblastic differentiation, formation of bone islets, and wound healing model induced by scratch assay.

Materials and Methods

Mesenchymal Stem Cell Culture

Adipose-derived MSC (ADMSC) that were cryopreserved in the second passage at -80°C were retrieved in vials and warmed to room temperature in a 37°C water bath. Subsequently, the cells were placed in an α-MEM culture medium including 15% fetal bovine serum (FBS), 50 Ìgr/ml gentamycin, 100 UI/ml penicillin, 100 UI/ml streptomycin, and 100 UI/ml amphotericin to promote cell proliferation and confluence and then were incubated at 37°C with 5% CO_2. The samples were morphologically evaluated and photographed under an inverted phase-contrast microscope.[30–32] The cells were then embedded in a frozen ADMSC culture and inoculated onto 12-well culture plates at 2.5×10^4 cells/cm^2.

MTT Assay

Cell viability and proliferation was assessed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay which is a widely used enzymatic test based on the cleavage of the tetrazolium ring of MTT by dehydrogenases. MTT is actively absorbed into living cells and the reaction is reduced to non-water-soluble blue-purple formazan crystals after being catalyzed by succinate dehydrogenases in the mitochondria. The amount of these crystals, which can be determined spectrophotometrically, serves as an estimate for the number of living cells in the sample. An MTT stock solution (5 mg of MTT/ml of distilled water) was prepared in sterile phosphate buffered saline (PBS) and was inoculated onto 96-well culture plates. After remaining in an incubator for 3 h, optical density was determined using an ELISA kit (Bio-Rad, ABD) at the 540 nm wavelength. The mean of absorbance values from control wells was accepted as the control absorbance value indicating 100% cell viability. The mean of absorbance values from the wells treated with solvents and agents was expressed as the percentage of the control value.[33]

Osteoblastic Differentiation and Characterization of ADMSC

The ADMSCs frozen at -196°C were recovered from fluid nitrogen and warmed to room temperature and then placed in culture medium for proliferation. The osteogenic factors were added with 50 mg/mL ascorbic acid, 10–8 M dexamethasone, 10 mM b-glycerol phosphate, and 10 nmol/L 17β-estradiol (E2) for osteoblastic differentiation. The cells in the second passage were used for the experiment were characterized as CD90-positive and CD45-negative by immunohistochemical staining. Confluent cultures were examined immunohistochemically using antibodies against OC (Biomedical Technologies, Stoughton, MA, USA), or ON (AON-1; Developmental Studies Hybridoma Bank, Stoughton, MA, USA) as follows. Samples were fi xed with 4% paraformaldehyde in PBS, pH 7.4. Endogenous peroxidase was inactivated by incubation with 3% H_2O_2 for 30 min. After incubation with primary antibodies, the sections were incubated with biotinylated secondary antibodies and reacted with peroxidase-conjugated streptavidin using the protocol of a Histostain kit (Zymed, San Francisco, CA). The primary antibody was omitted for negative control. Samples were then incubated with diaminobenzidine/hydrogen peroxide (00–2020, Invitrogen, Camarillo, CA, USA). Cells were counterstained with Mayer's hematoxylin (02274390059, J.T. Barker, Deventer, Holland). After washing in distilled water, cover glasses were removed from the plate, then they were reversed and mounted on to the slides with mounting medium (AML060, Scytek, UT, USA), then evaluated under a light microscope (Olympus BX40, Olympus Corp., Tokyo, Japan).

The osteoblastic differentiated from MSC at confluent level were observed as island of cells in mineralized matrix and stained by Alizarin Red S and Von Kossa. At the end of the incubation of canine BMSC, the cell layers were washed three times with ice-cold PBS, pH: 7.4, scraped off the plates into 300–500 ml ice-cold 0.1% Triton-X 100 and subjected to mild sonication to lyse the cells completely. ALP activity was measured using 2 mM p -nitrophenylphosphate (p-NPP) in assay buffer (0.1 M diethanolamine, 1 mM MgCl 2, pH 10.5) at 37°C for 10–60 min. ALP activity is expressed as nmol of p-nitrophenol/mg protein/h. Calcium phosphate deposit in the ECM can be detected by the Von Kossa (VK) method in which calcium phosphate deposits are stained brown to black. The media of confluent cultures were removed and the cells were washed once in cold PBS without Ca/Mg. Fixation was carried out for 15 min in 2.5% glutaraldehyde at room temperature and cultures were washed in distilled H_2O for 10 min. Distilled H_2O was removed and cells were covered with 5% AgNO 3 solution for 45 min. BMSC also were processed for AR staining of mineralized matrix. The cells were washed three times in distilled H_2O and allowed to air dry overnight. AR S (sodium 1, 2-dihydroxyanthraquinone3-sulfonate) was used as a colorimetric reagent for detection of calcium salts. Slides prepared from the tissue culture (Nunc® Lab-Tek® Chamber

Slide™ system; Sigma, Roskilde, Denmark) were fixed in 10% formalin for 24 h and exposed to 1% ARS solution (pH=4.2) for 5 min at room temperature. The cells were washed in distilled water for 30 min to remove unreacted reagent, then air dried and photographed.[34]

Inducing a Mimicked Model of Diabetic Osteoporosis and Fracture in Culture

Normal glucose with estrogen supplementation in medium was used as the control value for the bone matrix formation in culture. A high-glucose medium without estrogen supplementation was used to mimic osteoporosis in women with diabetic menopause. The bone matrix in culture was scratched with a sterile pipette tip and the effect of glucose and estrogen supplementation was evaluated by comparing with the control values. Healing was accepted as complete close of the wound area by the cells.[35,36]

Transferring Microscopic Images to Digital Media

Morphological observations were performed using an inverted phase-contrast microscope and the images were transferred to computer environment and evaluated.[33]

Transforming Histological Images to Digital Data

Closure of wound healing was evaluated as digital data by morphometric measurement of the interval. Immunohistochemical staining and intensity was obtained by H-score. H-Score=S Pi (i+1), where i is the intensity of staining with a value of (1), (2) or (3) (minimal, mild, moderate, or strong, respectively) and Pi the percentage of cells stained with each intensity, varying between 0–100%. Results were expressed as mean±SE. Differences among groups were statistically analyzed with one-way ANOVA where appropriate. A p value of 0.05 was considered as statistically significant. All the statistical analyses were performed using Graphpad (GraphPad Software Inc., La Jolla, CA, USA) software.[33]

Results

The MSC markers elicited *via* mechanical isolation of approximately 1 cm^2 of adipose tissue harvested from rat abdomen were characterized by immunostaining (**Figure 1**) as CD90-positive and CD45-negative (**Table 1**). These cells were added with ascorbic acid, b-glycerol phosphate, dexamethasone, and estrogen factors to allow osteoblastic differentiation and the cells were characterized together with their markers. In culture medium, islet-shaped bone matrix and calcium mineralization were identified *via* Alizarin Red S and Von Kossa staining (**Figure 2**). It was also noted that cells in the osteogenic medium supplemented with estrogen expressed the osteogenic markers starting from center to periphery of the islets.

Table 1
H-score of MSC characterized as CD90-positive and CD45-negative.

H-score	
CD90	**CD45**
234.45±22.68	35.22±16.48

Islet formation and the expression of MSC markers decreased significantly in the mimicked model of diabetic osteoporosis that was formed in culture medium by adding high glucose and removing estrogen (**Table 2**). The effects of MSC and niche were examined in the wound model formed by the scratch induced with a sterile pipette tip (**Figure 3**) and it was revealed that MSC and niche which was factors secreted by MSC into their 24-h medium, promoted wound healing. It was also observed that the healing was initiated through cell proliferation beginning on one side of the wound, followed by the reformation of the bony tissue. Additionally, it was also noted the administration of cell-based therapy accelerated wound closure and also achieved more favorable outcomes in terms of wound healing and expression of MSC markers when compared to untreated culture (**Table 3**).

Discussion

Diabetes is a chronic health problem leading to significant losses due to its complications. Diabetes-associated changes in bone microstructure and osteopenia can pose serious challenges for dentists and orthopedists.[37,38] Osteoporosis is a bone disease characterized by reduced bone mass and resistance and increased fragility and is known to affect most menopausal women. Osteoporosis becomes more significant when accompanied by diabetes.

Table 2
H-score of differentiated MSC to osteoblast which characterized by ON and OC staining.

	ON	OCN
Control (OM + E2 + NG)	228.36±28.44	244.86±26.08
Mimicked model (OM – E2 + HG)	124.22±24.88	142.18±23.96
Treatment (OM – E2 + HG + MSC + Niche)	174.16±22.24	195.14±26.34

E2: 17β-estradiol; **HG:** high glucose; **NG:** normal glucose; **Niche:** secretion of factors from MSC for 24 hours into medium; **OCN:** osteocalcin; **OM:** osteogenic medium; **ON:** osteonectin.

On the other hand, estrogen deficiency is a condition that leads to decreased osteoblastic function and regeneration capacity and delayed fracture healing. Accordingly, MSC therapies can be effective options for such conditions since MSC can be easily obtained and proliferated for therapeutic purposes and due to their immune tolerance property.[39] In the present study, a mimicked model of diabetic osteoporosis and fracture was formed in culture medium which allowed osteoblastic differentiation and bone islet formation. Moreover, the administration of MSC and niche (*i.e.* secreted factors) were found to have a therapeutic effect. The osteoblastic differentiation of

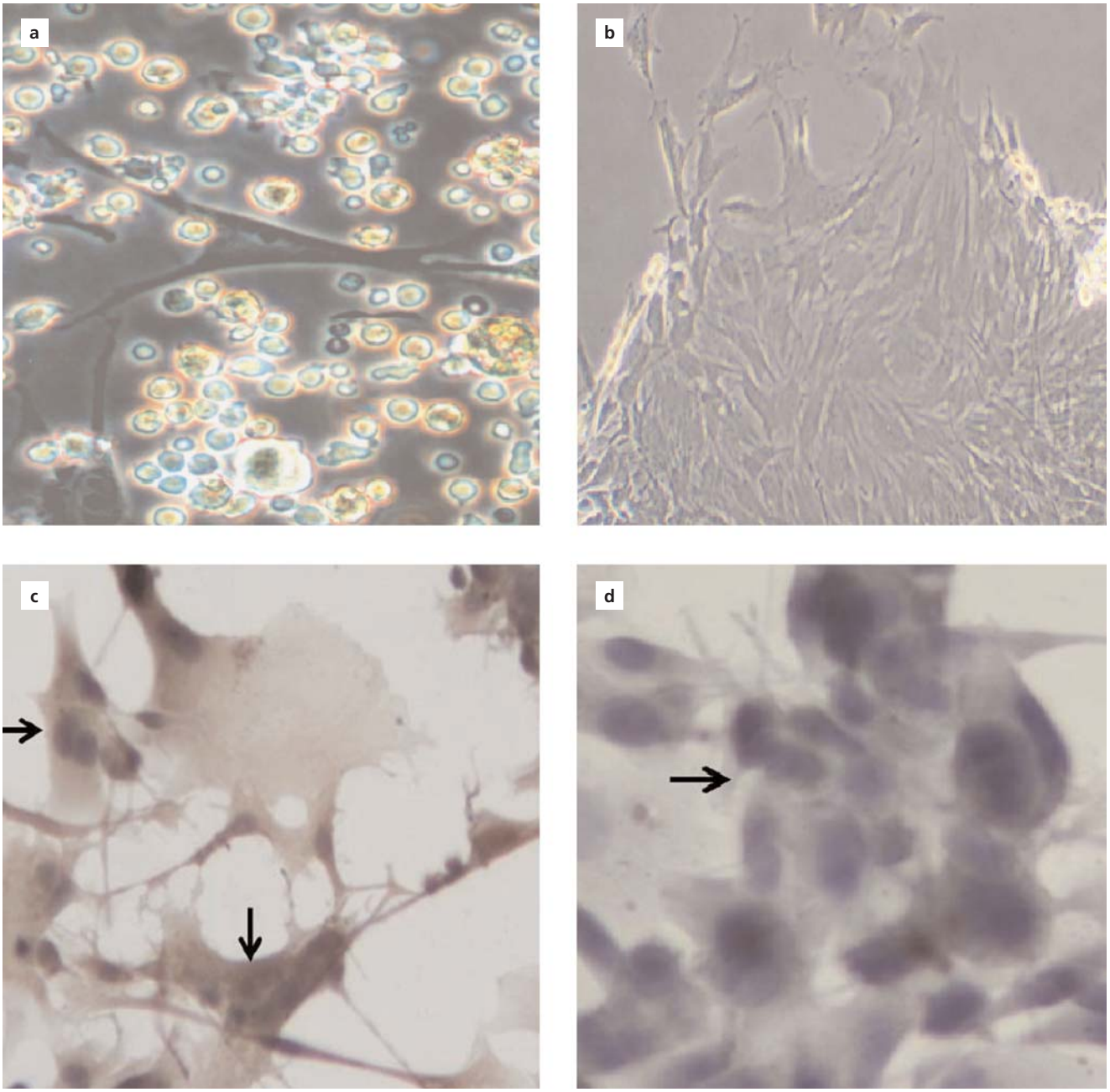

Figure 1. Morphological observation of MSC under phase contrast microscope and immunoperoxidase reactivity of CD90 for MSC which most of the cells stained and CD45 for hematopoietic cells which did not stain positive with antibody against CD45. (**a**) MSC and adipose tissue cells (×400); (**b**) Proliferation of MSC (×200); (**c**) CD90-positive (×400); (**d**) CD45-negative (×400). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

MSC in osteogenic culture with 17β-estradiol led to the formation of osteoblasts and bone islets, which was decreased significantly by the administration of a high-glucose medium and removal of estrogen. In a related manner, MSC and their niche significantly improved wound healing in the wound model induced by scratch assay.

The cells seeded in culture medium were confirmed as MSC that were characterized as CD90-positive and CD45-negative. Similarly, this mesenchymal characterization has been reported in previous studies and has also been confirmed by flow cytometry.[9–11] It was also revealed that the addition of estrogen into the osteogenic culture medium led to greater bone islet formation and osteoblastic differentiation. Moreover, studies have shown that inhibition of bone islet formation and osteoblastic differentiation in a high-glucose medium can be reversed by the addition of 17β-estradiol (E2).[37] In our study, a mimicked model of diabetic osteoporosis was formed in a high-glucose culture medium with E2 supplement and without estrogen and was used as control.[35] In this medium, a wound model was induced by the scratch assay, which is commonly used in fibroblast culture, and the model promoted significant wound healing. Additionally, as consis-

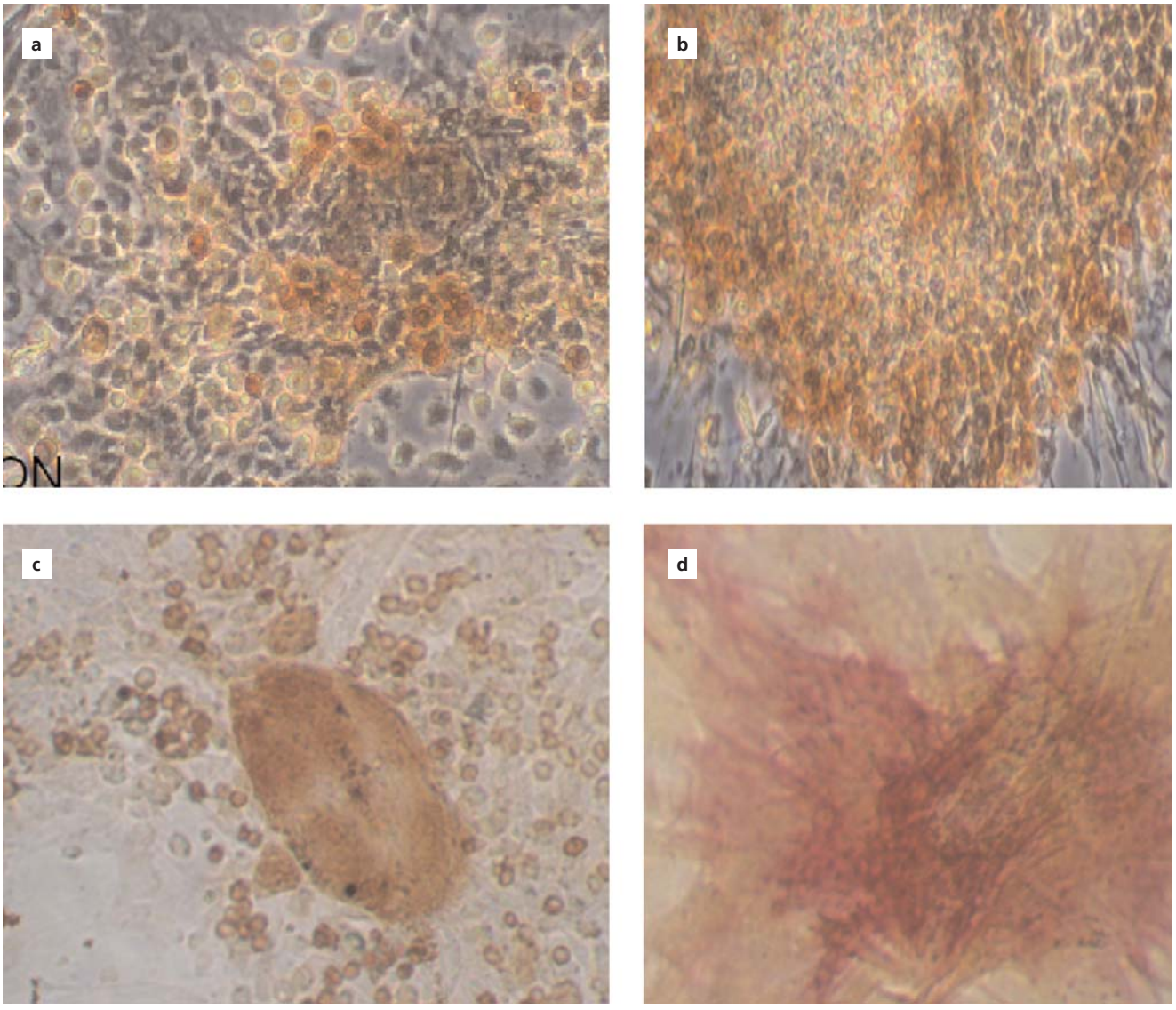

Figure 2. Osteoblast differentiation detected by osteonectin and osteocalcine staining. (**a**) Osteonectin; (**b**) Osteocalcin; (**c**) Von Kossa stain; (**d**) Alizarin Red S stain. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

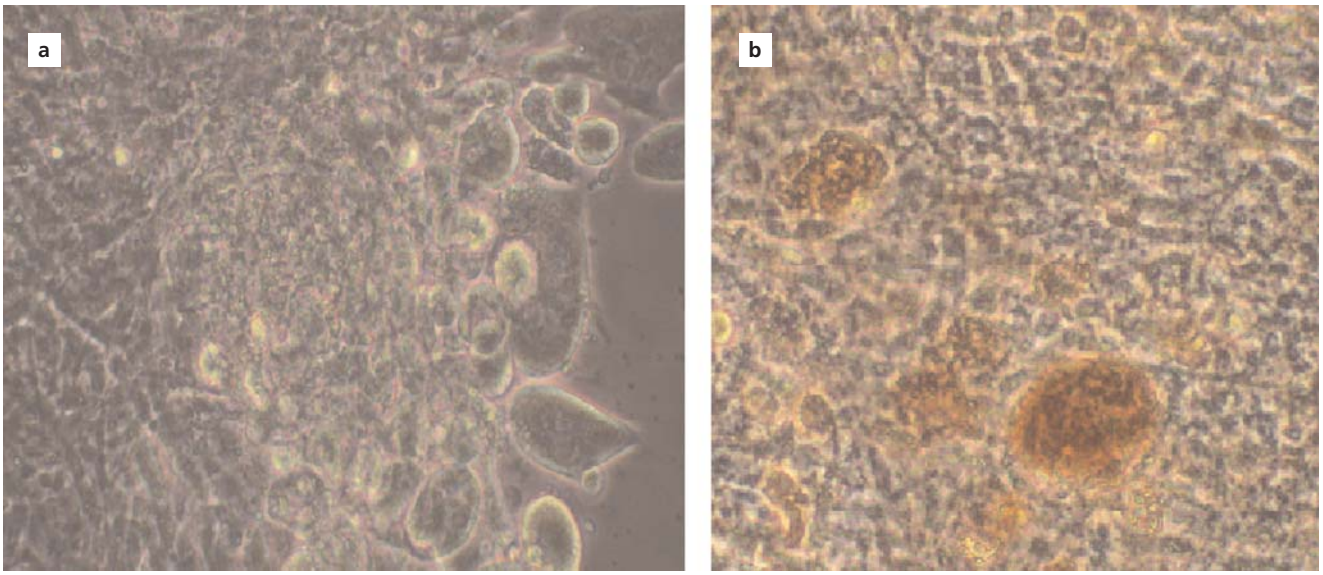

Figure 3. The wound model induced by scratch with pipette tip, where cell growth, differentiation and reformation occurred. The mimicked model of diabetic menopausal osteoporosis with high glucose and no estrogen supplement led to less healing compared to the control group. Wound healing in culture was followed by phase contrast microscope. (**a**) Wound area; (**b**) Reformation of bone matrix. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

tent with the literature, the addition of MSC and niche was found to have a contributory effect on the treatment.

The osteoblastic differentiation from MSC were found to induce mineralized bone matrix shown with Alizarin Red S and von Kossa staining. Similarly, this differentiation and the formation of mineralized bone matrix have also been shown by histochemical staining in previous studies.[37,39–41] All these results were consistent with the previous literature.

Osteogenesis is a complex process involving three phases: proliferation, matrix formation, and mineralization. RunX2 is a key marker of this process and is associated with the signaling pathways including Wnt/β-Catenin and PI3K/ AKT. Other markers include collagen I, osteonectin (ON), osteopontin (OPN) and RANKL has also been shown to be an immuno-histochemical marker of osteoblastic and osteoclastic differentiation.[42–44] In our study, the differentiation of MSC to osteoblasts in culture medium was detected by the immunohistochemical staining of ON and OCN. Similarly, this differentiation was also shown by staining of these two markers, as well as some other markers including OPN and bone morphogenetic proteins (BMPs).[14,45]

Scratch assay is commonly used in fibroblast culture for inducing wound models. However, to our knowledge, the use of scratch assay following the formation of bone matrix in culture has never been reported in the literature. Additionally, although this assay has been administered in skin wound healing with fibroblasts.[35,46–49] it has not been administered for osteoblasts. Accordingly, the present study is the first in the literature to show that the scratch assay as a repeatable experimental model and that MSC and their niche can be used for therapeutic purposes. In the present study, we investigated the effect of high-glu-

Table 3

Scratched area was measured between both edge of the wound at the end of the experiment. The administration of MSC and niche led to osteoblastic differentiation and bone matrix formation at a similar level to that of control group.

	Wound closure	Bone matrix formation	Osteoblast count
Control (OM + E2 + NG)	0.75±0.15 μm	65.45±25.15	25.12±8.24
Mimicked model (OM – E2 + HG)	0.33±0.12 μm	21.22±10.88	8.44±2.16
Treatment (OM – E2 + HG + MSC + Niche)	0.58±0.14 μm	45.37±18.56	15.62±5.72

cose culture medium with no estrogen supplement on wound healing and found that the osteoblasts differentiated from MSC did not induce bone matrix formation and had no significant effect on wound healing. Some previous studies also investigated the effect of high-glucose medium on experimental diabetes in culture.[20,50] In a similar way to our study, these studies also examined the effect of estrogen supplementation in culture on menopause condition and revealed that it had a favorable effect on osteoblasts.[29,45,51] Accordingly, our findings are consistent with the literature.

In our study, as shown by previous literature, the osteoblastic differentiation from MSC in an osteogenic medium with E2 supplement was found to express more ON and OC. Using high glucose decreased osteoblastic differentiation shown with ON and OC staning and reduced bone like island formation. Additionally, the removal of the E2 supplement that was used for mimicking osteoporosis further clarified the diabetes-induced damage and significantly decreased the expression of markers, which were also consistent with the literature. In contrast, the use of therapeutic MSC and niche increased the decreasing expression of markers. A previous study indicated that diabetic patients typically damaged osteoblasts and osteoclasts and also had an increased risk of oxidative stress, inflammation, delayed fracture healing associated with drug use, and decreased healing rates.[8]

A previous study reported that the osteoblastogenesis mineralization of rat bone marrow stromal cells is inhibited in hyperglycemic culture by activation of the Notch2 signaling pathway.[52] Another study evaluated diabetic rats induced by STZ and revealed that the transplantation of CXCL13-stimulated BMSC in culture increased cell proliferation rate and *in vivo* enhanced the corresponding ALP expression in bone healing.[16] Some other studies indicated that the administration of human diabetic serum into MSC culture medium lead to reduced osteogenic differentiation in association with high glucose levels and could also be an important factor for diabetic osteoporosis.[18] A study by Qu et al. examined the effect of miR-449 on osteogenic differentiation and its underlying mechanism in hBMSC using high glucose and free fatty acids treatment and revealed that the 14-day treatment dramatically decreased mineralization of hBMSC and resulted in impaired bone islets. The authors also noted that the miR-449 mimics decreased the protein expression levels of Runx2, ALP, collagen I, OCN, and BSP which were significantly increased by miR-449 inhibitors.[19] Another study investigated potential abnormalities of BMMSC in a rat menopause model established by ovariectomy and revealed that proliferation, migration, and differentiation of osteoclasts decreased, but increased by Wharton's jelly derived MSC.[20] In an experimental study, ADMSC were cultured to mimic diabetic osteoporosis and the results indicated that OPN and Runx2 expressions of ADMSC decreased and there was a noticeable reduction in mineralization, which was associated with DNA methylation and Wenti signal.[21] Likewise, some other studies established a rat diabetic osteoporosis model with STZ and high-fat diet and reported that administration of the model with plant extracts improved diabetic osteopenia and also prevented oxidative stress and apoptosis in the fracture induced in the femur.[22,23]

In the present study, a culture model was formed to investigate the effect of MSC and niche therapy on fractures that could result in death by embolies in patients with diabetic osteoporosis. This model could be a cost-effective method for the new treatment products to be applied in dental and orthopedic practice prior to animal experiments and clinical trials and also provides useful information for the drugs to be developed in future. To our knowledge, there are no studies conducted on MSC in the literature other than those investigating the effect of drugs and stem cell therapy on osteoblast differentiation.[9–11] Accordingly, the present study is the first of its kind to show that MSC and niche therapy leads to a significant improvement in impaired osteoblast function and in the reformation of bone matrix, particularly by increasing the number of osteoblasts, promoting bone matrix formation, and activating mineralization in culture. We consider that these findings can be used to produce a new treatment protocol in clinical practice.

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