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Metformin enhances the osteogenic activity of rat bone marrow mesenchymal stem cells by inhibiting oxidative stress induced by diabetes mellitus: an *in vitro* and *in vivo* study

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ABSTRACT

Purpose: The purpose of this study was to determine whether metformin (MF) could alleviate the expresssion of reactive oxygen species (ROS) and improve the osteogenic ability of bone marrow mesenchymal stem cells derived from diabetic rats (drBMSCs) *in vitro*, and to evaluate the effect of MF on the ectopic osteogenesis of drBMSCs in a nude mouse model *in vivo*.
Methods: BMSCs were extracted from normal and diabetic rats. *In vitro*, a cell viability assay (Cell Counting Kit-8), tests of alkaline phosphatase (ALP) activity, and western blot analysis were first used to determine the cell proliferation and osteogenic differentiation of drBMSCs that were subjected to treatment with different concentrations of MF (0, 50, 100, 200, 500 μM). The cells were then divided into 5 groups: (1) normal rat BMSCs (the BMSCs derived from normal rats group), (2) the drBMSCs group, (3) the drBMSCs + Mito-TEMPO (10 μM, ROS scavenger) group, (4) the drBMSCs + MF (200 μM) group, and (5) the drBMSCs + MF (200 μM) + H_2O_2 (50 μM, ROS activator) group. Intracellular ROS detection, a senescence-associated β-galactosidase assay, ALP staining, alizarin red staining, western blotting, and immunofluorescence assays were performed to determine the effects of MF on oxidative stress and osteogenic differentiation in drBMSCs. *In vivo*, the effect of MF on the ectopic osteogenesis of drBMSCs was evaluated in a nude mouse model.
Results: MF effectively reduced ROS levels in drBMSCs. The cell proliferation, ALP activity, mineral deposition, and osteogenic-related protein expression of drBMSCs were demonstrably higher in the MF-treated group than in the non-MF-treated group. H_2O_2 inhibited the effects of MF. In addition, ectopic osteogenesis was significantly increased in drBMSCs treated with MF.
Conclusions: MF promoted the proliferation and osteogenic differentiation of drBMSCs by inhibiting the oxidative stress induced by diabetes and enhenced the ectopic bone formation of drBMSCs in nude mice.

Keywords: Bone regeneration; Diabetes mellitus; Metformin; Oxidative stress; Stem cells

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Author Contributions
Conceptualization: Kai Dong, Zhong-Hao Liu; Formal analysis: Kai Dong, Zhong-Hao Liu; Methodology: Kai Dong, Wen-Juan Zhou; Project administration: Kai Dong, Wen-Juan Zhou, Zhong-Hao Liu; Writing - original draft: Kai Dong; Writing - review & editing: Kai Dong, Wen-Juan Zhou, Zhong-Hao Liu.

INTRODUCTION

Diabetes mellitus, a systemic metabolic disorder characterized by chronic hyperglycemia, is classified into types 1 and 2, as well as gestational diabetes mellitus. Type 2 diabetes mellitus accounts for 90%–95% of all diabetes cases [1]. Diabetes mellitus is closely related to periodontitis, which can result in the loosening and subsequent loss of teeth [2]. Hyperglycemia inhibits the formation of new bone and accelerates alveolar bone resorption associated with periodontitis [3]. In clinical practice, patients with diabetes often present with severe alveolar ridge resorption, and insufficient bone volume may pose a challenge for subsequent dental implant treatment [4].

Several surgical procedures have been developed to enlarge the alveolar crest. Autologous bone transplantation is the current "gold standard" for the reconstruction of jawbone defects, especially large bone defects [5]. However, the procedure has several major disadvantages such as a second surgical area, limited amounts of grafted bone, residual pain, nerve injury, and unpredictable resorption [6,7]. Notably, the use of intraoral autogenous bone blocks is not recommended for patients with diabetes because diabetes significantly increases the risk of graft failure in such cases [8]. Consequently, alternative methods using allogeneic bone, xenogeneic bone, and allogeneic bone have been proposed. Although these procedures have been proven to be effective techniques for the reconstruction of small bone defects, the management of larger bone defects remains a considerable surgical challenge [9].

Tissue engineering and therapy with stem cells could be an option for the preparation of bone grafts that promote the osseous healing of large defects [10]. Bone marrow mesenchymal stem cells (BMSCs) have been widely utilized in bone tissue engineering [11], and numerous studies have characterized the osteogenic potential of these cells [12,13]. However, in pathological conditions such as diabetes, the proliferation and osteogenic differentiation of BMSCs are significantly suppressed, which could lead to an imbalance of bone metabolism, finally leading to bone loss [14]. In diabetes, hyperglycemia leads to the accumulation of advanced glycation end products (AGEs) that generate reactive oxygen species (ROS) by binding the receptor for AGEs [15], which then activates the downstream signaling transduction pathway and affects bone metabolism [16]. ROS levels are closely related to bone metabolism and could significantly inhibit the osteogenic differentiation of BMSCs [17]. Therefore, the question of how to improve the osteogenic differentiation of BMSCs is an important aspect of stem cell treatment for bone defects in patients with diabetes.

Metformin (MF), an effective and safe hypoglycemic drug, is widely used to treat diabetes [18]. MF could promote osteoblast differentiation and bone formation by decreasing the intracellular ROS caused by hyperglycemia [19]. Additionally, MF enhanced the stemness and osteogenesis ability of alveolar bone BMSCs and accelerated new bone formation [20,21]. Despite its widespread use, the mechanism of action of this century-old medication is still not completely understood [22].

Thus, the aims of the present article are as follows: (1) to investigate whether MF could alleviate oxidative stress–induced damage and improve the osteogenic capacity of BMSCs derived from diabetic rats (drBMSCs) *in vitro*; and (2) to evaluate the effect of MF on the ectopic osteogenesis of drBMSCs in a nude mouse model *in vivo*.

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MATERIALS AND METHODS

Cell isolation, culture, and identification

First, a Sprague-Dawley rat model of type 2 diabetes mellitus was established using streptozotocin (Meilunbio, Dalian, China), according to the methods described by Reed et al. [23]. Then, rat BMSCs were obtained from the femurs and tibias of rats with type 2 diabetes mellitus and normal rats, respectively. Both the femurs and the tibias were harvested and the metaphysis on both sides was removed. The bone marrow cavities were flushed with complete Dulbecco's modified Eagle's medium (Meilunbio) containing 10% fetal bovine serum (Meilunbio) and supplemented with 1% penicillin/streptomycin (Meilunbio). After collecting the flushed liquid, the cell suspension was filtered through a 70 μM filter mesh (BD Biosciences, San Jose, CA, USA) and centrifuged for 5 minutes at 1,000 rpm. The cells were collected and incubated in 5% CO_2 saturated humidity at 37°C. Primary cultured cells were subjected to flow cytometric analysis to observe the expression of CD34, CD45, CD29, and CD90 (Biolegend, San Diego, CA, USA). Cell colonies were visualized with crystal violet staining (Meilunbio). Laser confocal microscopy was used to detect the expression of cytokeratin and vimentin (both obtained from ProteinTech, Wuhan, China). To demonstrate multipotent differentiation ability, rat BMSCs were cultured in osteogenic, adipogenic, and chondrogenic media (Cyagen Biosciences, Sunnyvale, CA, USA). After incubation for 10, 14, and 21 days, the cells were subjected to alizarin red, Oil red O and Alcian blue staining, respectively. Third- to fifth-generation cells were harvested for further experiments.

In vitro experiments

Cell treatment

To explore the effect of different concentrations of MF on cell proliferation and osteogenic differentiation, drBMSCs were cultured in high glucose medium (35.5 mM) and osteogenic induction medium that contained different concentrations (0, 50, 100, 200, and 500 μM) of MF (Sigma, St. Louis, MO USA), respectively. Cell viability was evaluated at 1, 3, and 5 days, alkaline phosphatase (ALP) activity was evaluated at 3 and 7 days, and the expressions of osteogenesis-related proteins (osteopontin [OPN], collagen type I [Col-1], and runt-related transcription factor 2 [Runx-2]) were examined at 10 days by western blotting.

To detect the effect of MF on ROS and the osteogenic differentiation of drBMSCs, cells were cultured in osteogenic medium and divided into 5 groups: (1) normal rat BMSCs (the nrBMSCs group), (2) the drBMSCs group, (3) the drBMSCs + MF group, (4) the drBMSCs + Mito-TEMPO (Mito, ROS antagonist, 10 μM, Sigma) group, and (5) the drBMSCs + MF + hydrogen peroxide (H_2O_2, ROS activator, 50 μM, Meilunbio) group. Intracellular ROS was assessed at 24 hours, senescence-associated β-galactosidase (SA-β-gal) positive cells were measured at 7 days, ALP activity and calcium nodules were examined by ALP and alizarin red staining at 10 and 21 days, respectively, and osteogenesis-related protein expression was evaluated at 10 and 21 days by western blotting and immunofluorescence assays.

Cell viability assay

A Cell Counting Kit-8 (CCK-8) kit (Meilunbio) was used. In brief, rat BMSCs were cultivated in 96-well plates (5×10^3 cells/well) for 1, 3, and 5 days. Then, the CCK-8 solution was added (10 μL/well) and incubated for 60 minutes at 37°C and 5% CO_2. A microplate reader (Bio-Rad, Hercules, CA, USA) was used to measure the absorbance at a 450-nm wavelength.

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ALP activity assay

An ALP activity detection kit (Beyotime, Shanghai, China) was used. Rat BMSCs were cultivated in 96-well plates (5×10^3 cells/well) for 3 and 7 days. Then, cells were lysed by RIPA lysate (Beyotime) and centrifuged at 14,000 × *g* for 5 minutes. The standards and samples were subsequently added to the wells, followed by the addition of assay buffer and the chromogen substrate. The reaction was stopped after 30 minutes at room temperature by adding 100 μL per well of stop solution. The absorbance was then measured at 405 nm using a microplate reader (Bio-Rad).

Western blot analysis

The rat BMSCs were seeded in 6-well plates (1×10^5 cells/well) and cultured for 10 days. Cells were lysed and the protein concentration was determined with a bicinchoninic acid kit (Beyotime). Proteins in equal amounts were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%, Meilunbio) and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, St. Louis, MO, USA). Blots were blocked with 10% milk (BD Biosciences) and incubated with primary antibodies at 4°C overnight. Then the coverslips were incubated with secondary antibody for 1 hour, and an enhanced chemiluminescence chromogenic substrate (Meilunbio) was used to visualize the bands. The following primary antibodies were used: OPN (1:500, ProteinTech), Col-1 (1:1,000, ProteinTech), Runx-2 (1:500, Affinity, Changzhou, China), and GAPDH (1:10,000, ProteinTech). Additionally, a horseradish peroxidase-conjugated secondary antibody (1:10,000, Golden Bridge, Beijing, China) was used.

Intracellular ROS detection

An ROS Assay Kit (Jiancheng Bioengineering, Nanjing, China) was used. Briefly, rat BMSCs were cultured in 24-well culture plates plated using cell-climbing slices at a density of 1×10^4 cells and cultured for 24 hours. The cells were incubated with DCFH-DA solution (10 μM) as a fluorescent probe for 50 minutes at 37°C, protected from light; then, the cells were incubated with DAPI-containing anti-fluorescence quenching tablets and observed under a fluorescence microscope, and the respective sections were photographed. The fluorescence intensity of ROS was analyzed using Image-Pro Plus version 6.0 (Media Cybernetics, Rockville, MD, USA).

SA-β-gal assay

An SA-β-gal Staining Kit (Solebao, Beijing, China) was used. Rat BMSCs were seeded in 24-well culture plates plated with cell-climbing slices at a density of 1×10^4 cells and cultured for 7 days. The cells were then fixed in β-galactosidase fixation solution at room temperature for 15 minutes and stained with the staining solution at 37°C overnight. The percentage of positive cells for SA-β-gal staining was analyzed.

ALP staining

An ALP staining kit (Beyotime) was used. Briefly, rat BMSCs were cultured in 24-well plates (1×10^4 cells/well) for 10 days. After cell fixation with ALP fixative solution for 5 minutes, each well was incubated for 2 hours in the dark at room temperature with 100 μL of BCIP/NBT substrate. The cells were then viewed under a microscope and the staining intensity was quantified with Image-Pro Plus version 6.0 (Media Cybernetics).

Mineralization assay

Rat BMSCs were cultivated in 24-well plates (1×10^4 cells/well) for 21 days. After 30 minutes of paraformaldehyde fixation (4%, Meilunbio), the cells were then stained with 2% Alizarin

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Red S (Sigma) at room temperature for 20 minutes. Quantification was performed by treating the stained calcium nodules with 10% cetylpyridinium chloride solution (Sigma). Microplate readers (Bio-Rad) were used to measure the absorbance at 570 nm.

Immunofluorescence assay

After 21 days in culture, the rat BMSCs were fixed with 4% paraformaldehyde (Meilunbio) for 15 minutes, permeabilized with 0.5% Triton X-100 (Meilunbio) for 5 minutes, blocked with 10% goat serum (Golden Bridge) for 30 minutes, and stained with primary antibodies and stored overnight at 4°C. The cells were then incubated with secondary antibodies for 1 hour at room temperature, and the nuclei were stained with DAPI. The antibodies used in the immunofluorescence assay were as follows: osteocalcin (OCN, 1:500, ProteinTech) and a horseradish peroxidase-conjugated secondary antibody (1:10,000, Golden Bridge).

In vivo experiments

Animals

The ectopic osteogenesis model was established by using male nude mice (5 weeks old, purchased from Jinan PengYue Laboratory Animal Breeding Co., Ltd., Jinan, China) to assess the osteogenesis of rat BMSCs. The experimental procedure followed the ARRIVE guidelines and was approved by the Animal Care Committee of the Yantai Stomatological Hospital Affiliated to Binzhou Medical College, China (No. 2020-22). Based on the 3R (replacement, reduction, and refinement) principle, the number of animals could be effectively minimized by using 2 implantation sites per animal. As determined from preliminary experiments, a sample size analysis with PASS software V.15.0.1 (NCSS, LLC, Kaysville, UT, USA) showed that 24 animals would be necessary to obtain a power of 80% with a significance level (α) of 5%. Animals were randomly divided into 4 groups (n=6/group): (1) a blank control group (only the hydroxyapatite scaffolds, which were purchased from Shaanxi Reshine Biotech Co., Ltd [Xian, China], were implanted subcutaneously in the dorsum of the nude mice), (2) the nrBMSCs group (nrBMSCs were seeded onto the surface of hydroxyapatite scaffolds and then implanted subcutaneously in the dorsum of the nude mice), (3) the drBMSCs group (hydroxyapatite scaffolds loaded with drBMSCs were implanted subcutaneously in the nude mice); (4) the drBMSCs + MF group (hydroxyapatite scaffolds loaded with drBMSCs were implanted subcutaneously in the nude mice, and 200 μM MF was injected locally once every 2 days for 5 weeks).

Surgical procedures

The nude mice were kept in specific pathogen-free environment at 20°C–23°C, at 42%–52% humidity, with a 12-hour light-dark cycle. The mice were housed in individually ventilated cages, with a maximum of 4 mice per cage, and given water and food ad libitum. The mice fasted for 12 hours before surgery and had free access to water. The animals were anesthetized by intraperitoneally injecting 4% chloral hydrate (0.1 mL/10 g) until unconsciousness was established, as confirmed by the toe-pinch test. For subcutaneous implantation of the scaffolds loaded with rat BMSCs, 2 1-cm incisions were made along the male nude mice's left/right flanks. Pouches were made on both sides of the incision with blunt dissection, and the scaffolds and rat BMSCs were implanted according to the grouping as described above. The mice received adequate analgesia and prophylactic antibiotics in the immediate postoperative period. In each group, the activity, dietary intake, and wound healing of the mice were observed. After surgery, mice that displayed signs of infection were excluded from the study.

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Histological analysis

Mice were sacrificed after 6 weeks, and the ectopic bone was harvested and fixed in 4% paraformaldehyde. Subsequently, the samples were decalcified in 10% EDTA (pH 7.4) for 15 days and embedded in paraffin after dehydration. Serial sections (4 mm thick) were stained with hematoxylin and eosin (H&E) and Masson trichrome. Before the evaluation, the examiner of the slides was carefully trained, and measurements were taken in triplicate. The area of new bone formation was quantified as a percentage using Image J version 1.8.0 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Descriptive statistics are presented as mean ± standard deviation. The Kolmogorov-Smirnov test was used to assess normality. Data with a normal distribution were compared using the independent-sample *t*-test, while data with a non-normal distribution were compared using the non-parametric Mann-Whitney test. For multi-group comparisons, data with a normal distribution were assessed using 1-way analysis of variance, followed by the least-significant difference *post hoc* test. Data with a non-normal distribution were assessed using the Kruskal-Wallis test. All statistical analyses were performed with SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). The level of significance was set at $P<0.05$.

RESULTS

Characterization of BMSCs obtained from normal and diabetic rats

Flow cytometry assessments revealed higher expression levels of CD29 and CD90 both in nrBMSCs and drBMSCs, but almost none expressed CD34 and CD45 (**Figure 1A**). In the immunofluorescence analyses, all cells expressed vimentin, while no cytokeratin expression was detected (**Figure 1B**). Crystal violet staining revealed the typical spindle shape of a single BMSC, as well as the whirlpool arrangement type of BMSC colonies (**Figure 1C**). After osteogenic induction of BMSCs for 21 days, alizarin-red-positive calcium nodules were observed. Following 10 days of adipogenic induction, lipid droplets were observed. Moreover, Alcian blue staining demonstrated that the chondrogenic medium induced chondrogenesis at 14 days (**Figure 1D**).

Effects of different concentrations of MF on drBMSCs

The CCK-8 assay demonstrated that the absorbance values among all groups showed no significant differences on day 1. On days 3 and 5, the absorbance value increased gradually with increasing MF concentrations; however, when the MF concentration was higher than 200 μM, absorbance decreased (**Figure 2A**). In the ALP activity assays, drBMSCs on days 3 and 7 showed increased ALP activity with increasing MF concentrations; however, when the MF concentration increased above 200 μM, the value dropped (**Figure 2B**). Similarly, western blot analysis demonstrated that with increasing MF concentrations, the expression of osteogenic-related proteins in drBMSCs on day 10 increased gradually until the MF concentration reached 200 μM (**Figure 2C**). Consequently, 200 μM MF was selected for subsequent experiments.

MF protected rat BMSCs from decreased osteogenic ability caused by diabetes-induced oxidative stress

As shown in **Figure 3A**, the ROS levels were significantly higher in drBMSCs than in nrBMSCs; MF significantly reduced the ROS levels of drBMSCs, similar to Mito; however, as H_2O_2 was added, the effect of MF was repressed and ROS concentrations significantly increased. Similarly, the SA-β-gal staining showed that MF could significantly reduce the cellular

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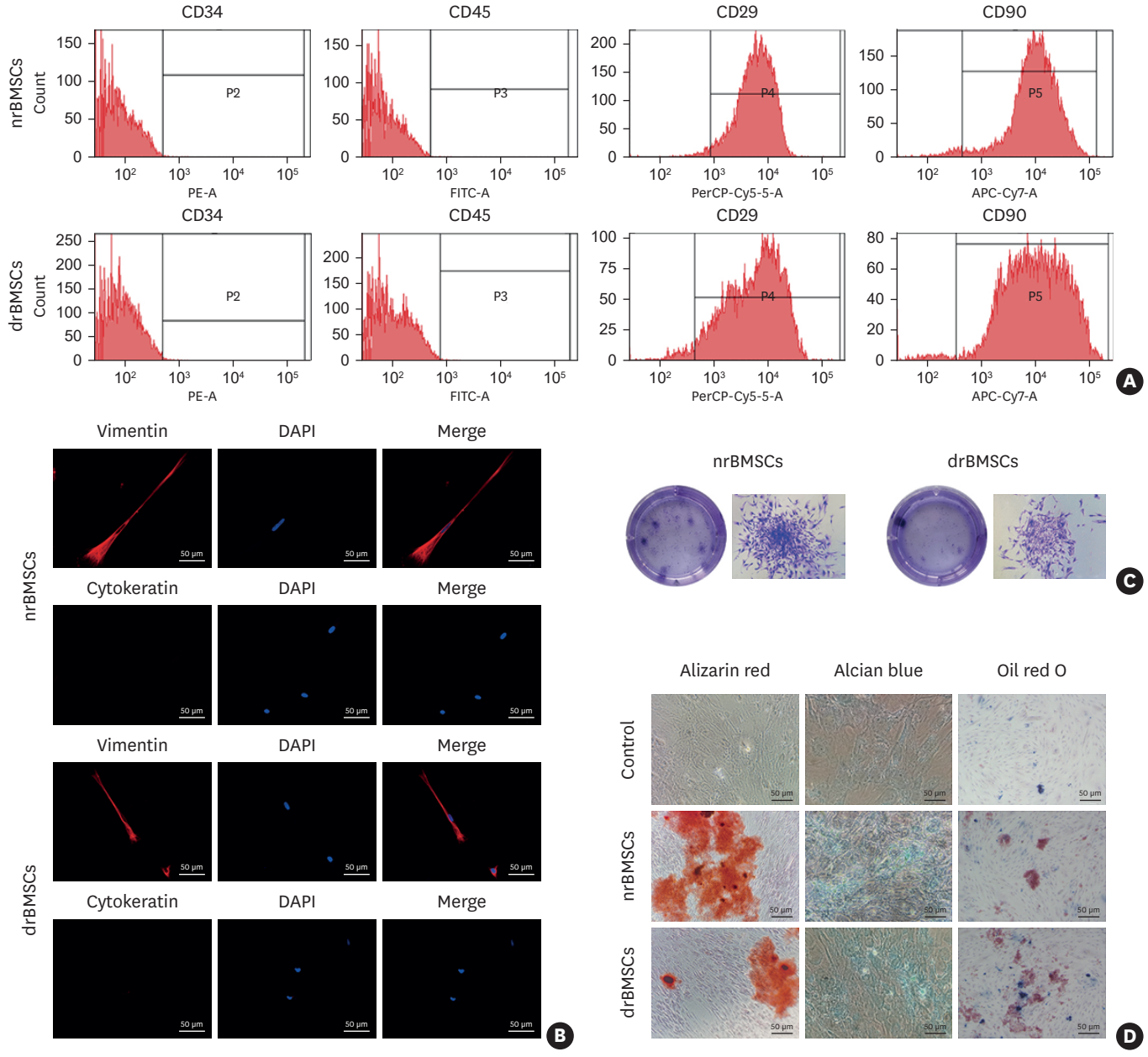

Figure 1. Identification of rat BMSCs. (A) Flow cytometry analysis of the cell surface markers. (B) Immunofluorescence expression of vimentin and cytokeratin. (C) Cell colonies stained with crystal violet. (D) Osteogenic, chondrogenic, and adipogenic differentiation were assayed.
BMSCs: bone marrow mesenchymal stem cells, nrBMSCs: BMSCs derived from normal rats, drBMSCs: BMSCs derived from diabetic rats.

senescence induced by diabetes, but adding H_2O_2 suppressed the effect of MF (**Figure 3B**). The specific data are presented in **Table 1**.

As shown in **Figure 4A and B**, ALP and alizarin red staining analysis demonstrated that MF-stimulated drBMSCs produced more ALP activity and mineralized matrix than MF-unstimulated drBMSCs. However, H_2O_2 still obstructed the pro-osteogenic effects of MF. The immunofluorescence (**Figure 4C**) and western blot (**Figure 4D**) assays showed that the protein expression of OPN, Col-1, Runx-2, and OCN in drBMSCs was significantly promoted by MF; similarly, H_2O_2 inhibited the effects of MF. The specific data are presented in **Table 2**.

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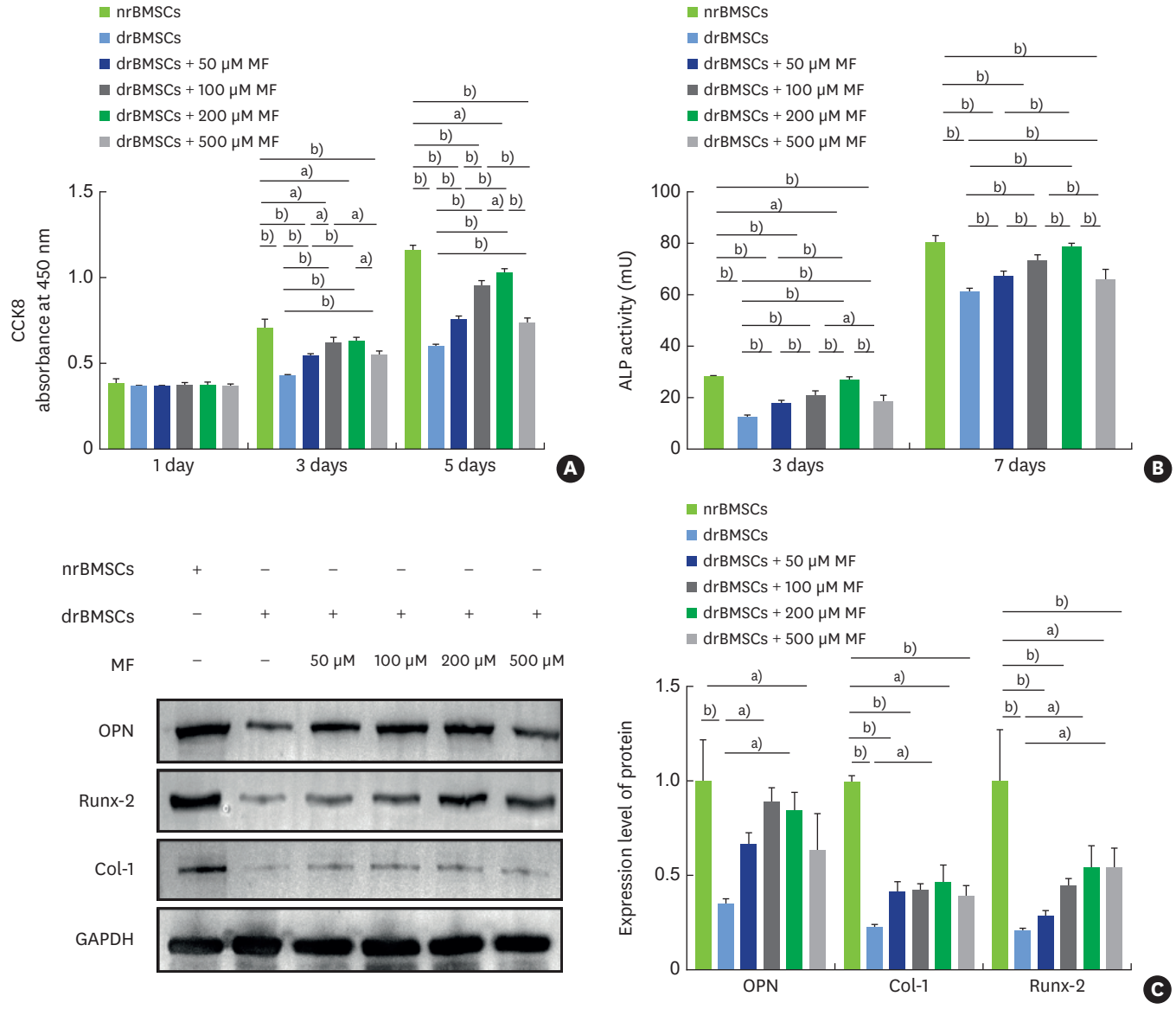

Figure 2. The effects of different concentrations of MF on proliferation and osteogenic differentiation of drBMSCs. (A) CCK-8 analysis at 1, 3, and 5 days. (B) ALP enzyme activity analysis at 3 and 7 days. (C) Western blot assay of the protein expression of OPN, Runx-2, and Col-1 at 10 days.
MF: metformin, drBMSCs: bone marrow mesenchymal stem cells derived from diabetic rats, CCK-8: Cell Counting Kit-8, ALP: alkaline phosphatase, nrBMSCs: bone marrow mesenchymal stem cells derived from normal rats, OPN: osteopontin, Runx-2: runt-related transcription factor 2, Col-1: collagen type I.
[a]$P<0.05$; [b]$P<0.01$.

MF enhanced *in vivo* ectopic bone formation of drBMSCs in nude mice

The H&E and Masson trichrome staining results indicated no bone regeneration in the blank control group. The nrBMSCs group showed more formation of bone tissues and more maturation of collagen, while the drBMSCs group showed significantly reduced new bone formation. The drBMSCs + MF group had higher new bone and mature collagen than the drBMSCs group (**Figure 5B and C**). Analyses of quantitative data showed significantly less new bone formation in diabetic rats than in the normal group; although MF efficiently stimulated the growth of new bone in diabetic rats, it did so to a significantly lesser extent than in the normal group (**Table 3**).

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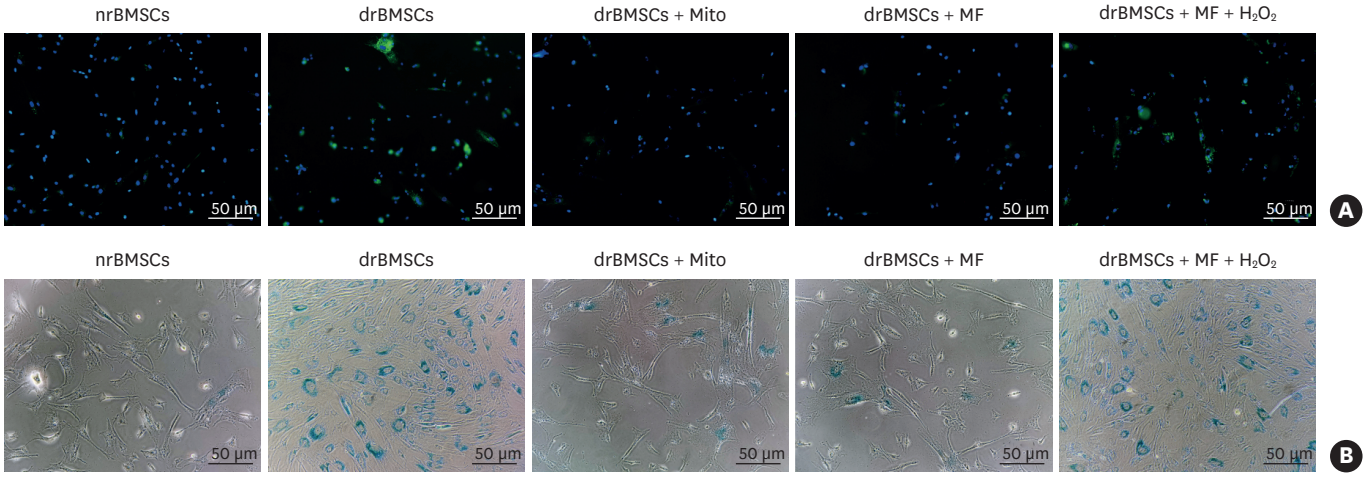

Figure 3. Effect of MF on ROS and SA-β-gal-positive expression of drBMSCs. (A) ROS fluorescence expression of drBMSCs on 24 hours. (B) The ratio of SA-β-gal positive cells at 7 days.
MF: metformin, ROS: reactive oxygen species, SA-β-gal: senescence-associated β-galactosidase, nrBMSCs: bone marrow mesenchymal stem cells derived from normal rats, drBMSCs: bone marrow mesenchymal stem cells derived from diabetic rats, Mito: Mito-TEMPO (ROS scavenger), H_2O_2: hydrogen peroxide (ROS activator).

Table 1. Inhibitory effect of MF on ROS and SA-β-gal expression of drBMSCs

Characteristics	nrBMSCs	drBMSCs	drBMSCs + Mito	drBMSCs + MF	drBMSCs + MF + H_2O_2
ROS (fluorescence intensity)	7.3847±1.51186	28.2643±1.51103[a] (P<0.01)	9.1717±1.75193[b] (P<0.05)	8.865±1.03652[b] (P<0.05)	31.6647±2.00613[a,c,d] (P<0.01, P<0.01, P<0.01)
SA-β-gal (positive cells, %)	2.3968±0.63947	39.5433±0.53995[a] (P<0.01)	7.7565±0.52188[b] (P<0.01)	6.9296±2.17322[b] (P<0.01)	43.3095±3.64541[a,c,d] (P<0.01, P<0.01, P<0.01)

MF: metformin, ROS: reactive oxygen species, SA-β-gal: senescence-associated β-galactosidase, drBMSCs: bone marrow mesenchymal stem cells derived from diabetes rats, nrBMSCs: bone marrow mesenchymal stem cells derived from normal rats, Mito: Mito-TEMPO (ROS scavenger), H_2O_2: hydrogen peroxide (ROS activator).
[a]Statistically significant from nrBMSCs group; [b]Statistically significant from drBMSCs group; [c]Statistically significant from drBMSCs + Mito group; [d]Statistically significant from drBMSCs + MF group.

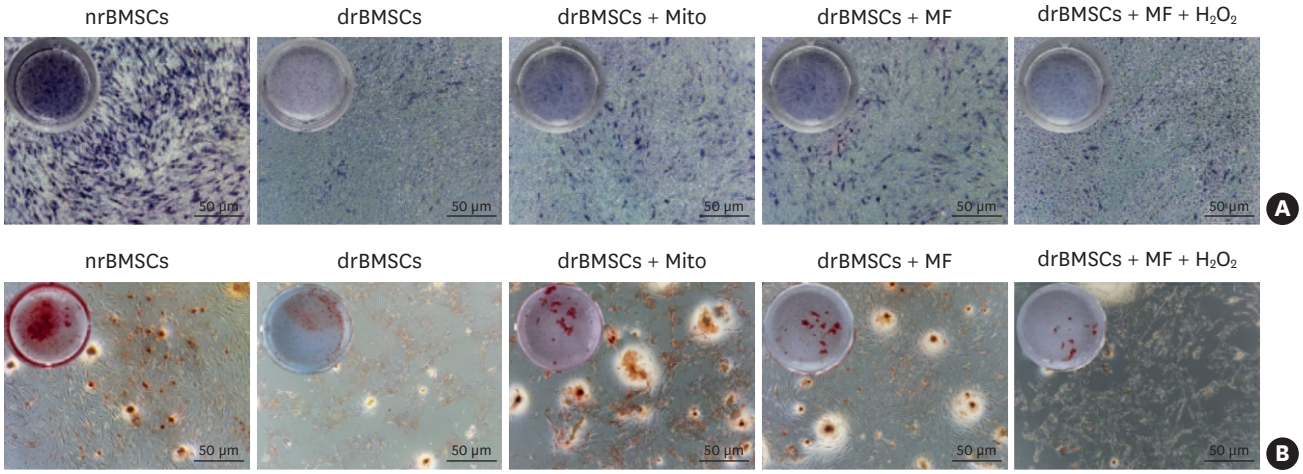

Figure 4. MF improved the osteogenic capacity of drBMSCs by modulating ROS. (A) ALP staining analysis at 10 days. (B) Alizarin red staining analysis at 21 days. (C) Immunofluorescence staining assay of the protein expression of OCN at 21 days. (D) Western blot assay of the protein expression of Col-1, Runx-2, and OPN at 10 days.
MF: metformin, drBMSCs: bone marrow mesenchymal stem cells derived from diabetic rats, ROS: reactive oxygen species, ALP: alkaline phosphatase, OCN: osteocalcin, OPN: osteopontin, Runx-2: runt-related transcription factor 2, Col-1: collagen type I, nrBMSCs: bone marrow mesenchymal stem cells derived from normal rats, Mito: Mito-TEMPO (ROS scavenger), H_2O_2: hydrogen peroxide (ROS activator).
[a]P<0.05; [b]P<0.01.

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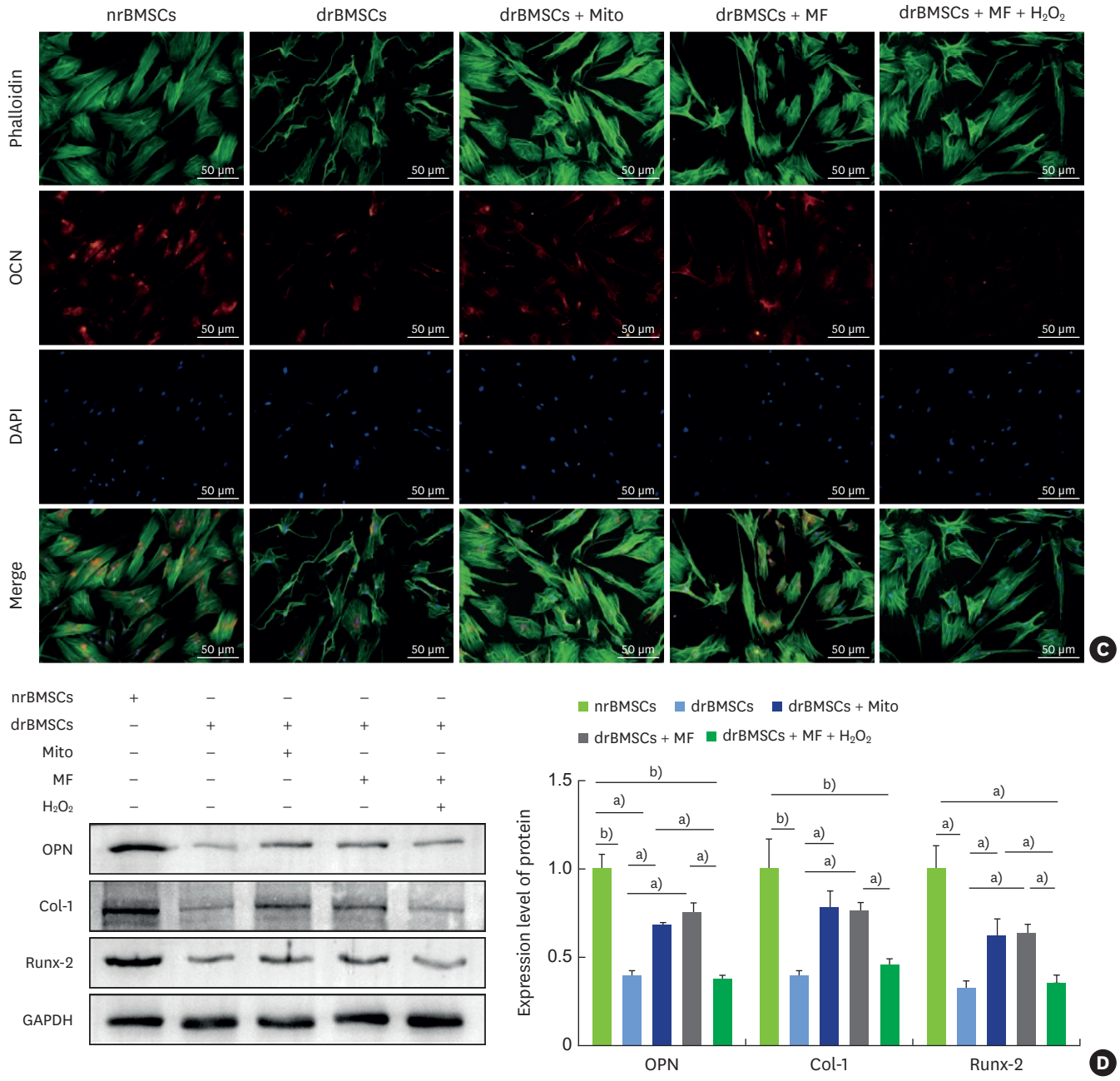

Figure 4. (Continued) MF improved the osteogenic capacity of drBMSCs by modulating ROS. (A) ALP staining analysis at 10 days. (B) Alizarin red staining analysis at 21 days. (C) Immunofluorescence staining assay of the protein expression of OCN at 21 days. (D) Western blot assay of the protein expression of Col-1, Runx-2, and OPN at 10 days.
MF: metformin, drBMSCs: bone marrow mesenchymal stem cells derived from diabetic rats, ROS: reactive oxygen species, ALP: alkaline phosphatase, OCN: osteocalcin, OPN: osteopontin, Runx-2: runt-related transcription factor 2, Col-1: collagen type I, nrBMSCs: bone marrow mesenchymal stem cells derived from normal rats, Mito: Mito-TEMPO (ROS scavenger), H_2O_2: hydrogen peroxide (ROS activator).
[a]$P<0.05$; [b]$P<0.01$.

DISCUSSION

The recruitment and differentiation of osteoprogenitor cells have been shown to be critical for bone regeneration, and BMSCs may play a significant role in this process [24]. As a metabolic disorder, diabetes mellitus could affect the proliferation and osteogenic

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Table 2. Stimulatory effect of MF on osteogenic differentiation of drBMSCs

Characteristics	nrBMSCs	drBMSCs	drBMSCs + Mito	drBMSCs + MF	drBMSCs + MF + H_2O_2
ALP Staining (average optical density)	36.6466±2.4962	12.181±0.84934[a] (P<0.01)	29.75±0.49814[b] (P<0.05)	27.3362±1.17337[b] (P<0.05)	12.8707±1.2557[a,c,d] (P<0.01, P<0.01, P<0.01)
Alizarin red staining (optical density)	2.8179±0.04057	0.9988±0.06732[a] (P<0.01)	1.9412±0.05254[b] (P<0.05)	1.8079±0.07725[b] (P<0.05)	1.294±0.09433[a,c,d] (P<0.01, P<0.05, P<0.05)
OCN expression (fluorescence intensity)	40.7184±3.02658	19.1544±1.28609[a] (P<0.01)	29.8732±1.20002[b] (P<0.05)	27.2372±0.40103[b] (P<0.05)	16.8423±2.42755[a,c,d] (P<0.01, P<0.01, P<0.01)

MF: metformin, drBMSCs: bone marrow mesenchymal stem cells derived from diabetes rats, nrBMSCs: bone marrow mesenchymal stem cells derived from normal rats, Mito: Mito-TEMPO (reactive oxygen species scavenger), H_2O_2: hydrogen peroxide (reactive oxygen species activator).
[a]Statistically significant from nrBMSCs group; [b]Statistically significant from drBMSCs group; [c]Statistically significant from drBMSCs + Mito group; [d]Statistically significant from drBMSCs + MF group.

differentiation of BMSCs [25]. Likewise, our experiments revealed that the osteogenic ability of BMSCs in diabetic rats was significantly lower than that of normal rats. Therefore, exploring the best means for BMSCs to differentiate under diabetic conditions is essential for the success of bone regeneration.

This present study found that diabetes mellitus significantly inhibited the proliferation, osteogenic differentiation, and mineralization of rat BMSCs. Hyperglycemia may cause these effects due to increased generation of ROS, and it has been reported to induce mitochondrial dysfunction leading to increased production of ROS [26]. Although ROS are specific signaling molecules that regulate multiple pathways, excessive accumulation of intracellular ROS can lead to oxidative damage to biomolecules and organelles [27]. According to studies, oxidative stress inhibits osteoblast differentiation, induces osteoblast insults, and causes apoptosis of osteoblasts [28]. In eukaryotic cells, mitochondrial oxidative phosphorylation is the primary source of ROS production [29]. Research has shown that mitochondria and ROS play a critical role in modulating bone cells, and a disruption in mitochondrial homeostasis caused by diabetes is involved in abnormal bone metabolism [30,31]. Additionally, this study found that diabetes mellitus increased ROS production in rat BMSCs, but Mito significantly inhibited ROS production and enhanced osteogenesis in drBMSCs. Therefore, interventions to improve mitochondrial function may be helpful for improving the bone-forming capacity of BMSCs under diabetic conditions.

MF is a first-line oral hypoglycemic widely used in managing diabetes mellitus [32]. Several studies have shown that MF, in addition to its hypoglycemic effect, has many beneficial effects on osteogenesis by promoting osteoblast precursors and mesenchymal stem cells [33]. This study found that MF inhibited ROS production while promoting drBMSC proliferation and osteogenic differentiation. However, this effect was attenuated by H_2O_2, which implies that MF exerted its pro-osteogenic effects by regulating ROS levels. The inhibitory effect of MF on ROS production is one of its primary mechanisms. MF directly inhibits complex I of the mitochondrial electron transport chain, thereby affecting mitochondria-induced oxidative stress by reducing ROS [34]. In addition, we found that MF had a concentration-dependent effect on the proliferation and osteogenic differentiation of drBMSCs. This effect of MF gradually increased as MF concentrations increased from 0 to 200 μM but decreased at concentrations between 200 and 500 μM. The 200 μM concentration appeared to be a turning point. However, in previous studies, the best concentrations for other stem cell types varied widely [35,36]. Diverse conclusions may be reached for different cell types and experimental conditions. Hence, the effect and mechanism of MF on cell proliferation are complex and require further investigation. In addition, we found that MF prevented oxidative stress-induced senescence in drBMSCs, which is consistent with previously published research [37].

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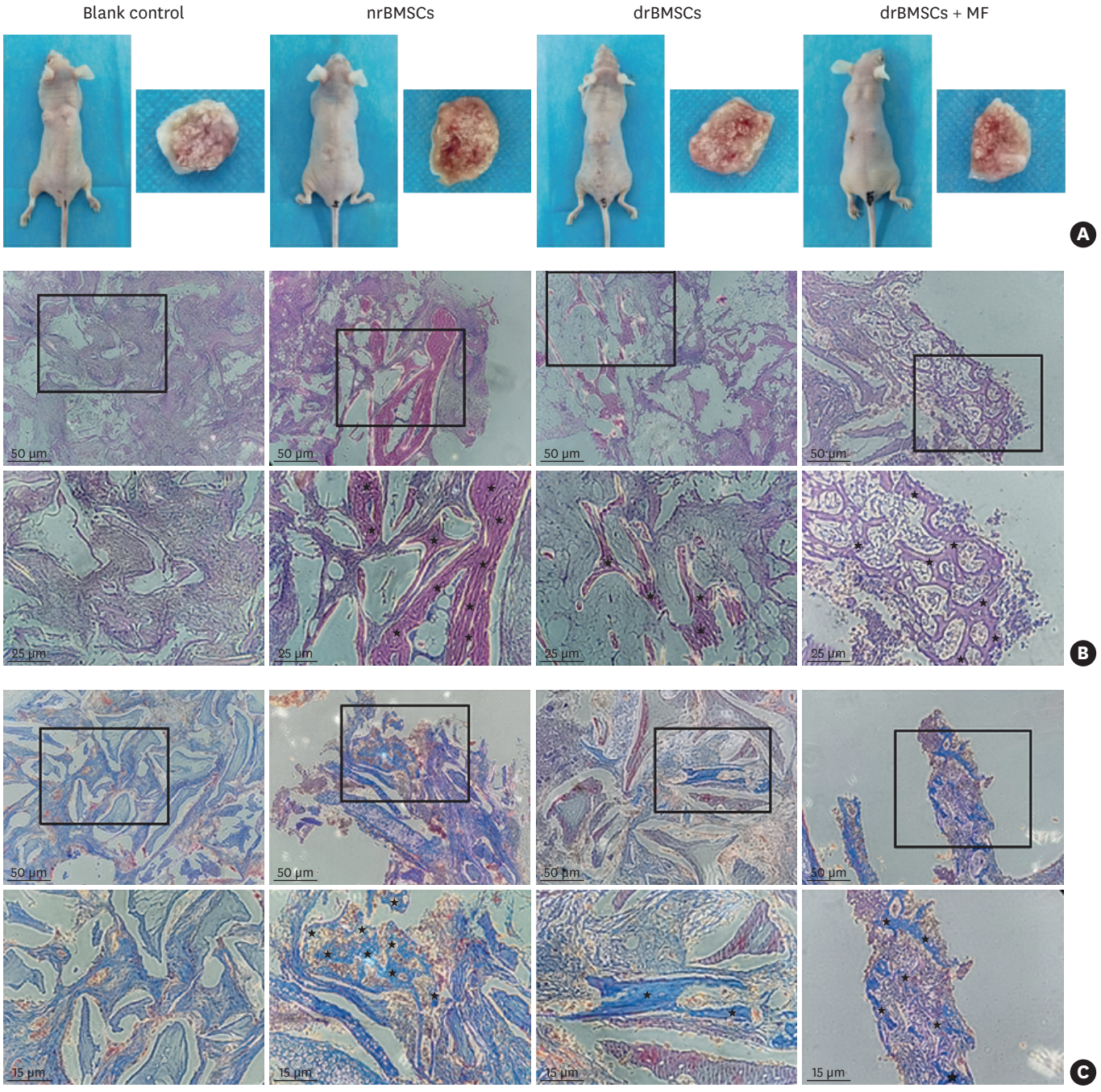

Figure 5. Ectopic bone formation assay in nude mice at 6 weeks to evaluate the effect of MF on drBMSCs. (A) macroscopic observation of the specimens. (B) H&E staining analysis. (C) Masson trichrome staining analysis (black box: region of interest, black asterisk: new bone tissue).
MF: metformin, nrBMSCs: bone marrow mesenchymal stem cells derived from normal rats, drBMSCs: bone marrow mesenchymal stem cells derived from diabetes rats, H&E: hematoxylin and eosin.

In agreement with the *in vitro* findings, H&E and Masson trichrome staining indicated that MF significantly facilitated the osteogenic differentiation of drBMSCs in the ectopic osteogenesis nude mouse model. As displayed in **Figure 5**, the newly formed bone in the MF-treated group appeared immature. It exhibited a trabecular pattern, while the normal group showed a more mature and dense bone structure. Consequently, although MF could

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Table 3. Histomorphometric analysis (%) of ectopic osteogenesis of drBMSCs

Characteristics	Blank	nrBMSCs	drBMSCs	drBMSCs + MF
H&E staining	0±0	2.5637±0.32779[a)]	0.6565±0.09053[a,b)]	1.0875±0.13003[a,b,c)]
(new bone formation, %)		(P<0.01)	(P<0.01, P<0.01)	(P<0.01, P<0.01, P<0.05)
Masson staining	0±0	2.4715±0.1435[a)]	0.5365±0.04523[a,b)]	0.9686±0.14103[a,b,c)]
(new bone formation, %)		(P<0.01)	(P<0.01, P<0.01)	(P<0.01, P<0.01, P<0.01)

nrBMSCs: bone marrow mesenchymal stem cells derived from normal rats, drBMSCs: bone marrow mesenchymal stem cells derived from diabetes rats, MF: metformin, H&E: hematoxylin and eosin.
[a)]Statistically significant from Blank group; [b)]Statistically significant from nrBMSCs group; [c)]Statistically significant from drBMSCs group.

efficiently enhance the osteogenesis of drBMSCs *in vivo*, it could not reach normal levels. This might be because diabetes inhibits osteogenesis through quite complex mechanisms, and the potential benefits of MF on bone metabolism in diabetes require further investigation. A limitation of this study was the lack of use of a jawbone defect model in animal experiments. Further research is needed to determine the specific mechanisms involved.

Our findings indicate that MF stimulated osteogenic differentiation of drBMSCs *in vitro* by inhibiting oxidative stress induced by diabetes mellitus. Furthermore, MF increased the formation of ectopic bone in drBMSCs *in vivo*. Diabetes mellitus is effectively treated with MF, which is a safe and cost-effective modality. This study may have clinical value in terms of improving the effect of bone reconstruction of alveolar bone defects in patients with diabetes.

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