Original Article

Synergistic effect of metformin and vitamin D₃ on osteogenic differentiation of human adipose tissue-derived mesenchymal stem cells under high D-glucose conditions

Nhi Nguyen-Yen Ha [a, b, c], Thi Kim Tan Huynh [b, c], Ngoc Uyen Phuong Phan [d], Thi-Hiep Nguyen [a, c], Long Binh Vong [a, c, **], Nhu-Thuy Trinh [a, c, *]

[a] School of Biomedical Engineering, International University, 700000 HCMC, Viet Nam
[b] Faculty of Biology and Biotechnology, University of Science, 700000 HCMC, Viet Nam
[c] Vietnam National University-Ho Chi Minh City (VNU-HCMC), 700000 HCMC, Viet Nam
[d] Biotechnology Center of Ho Chi Minh City, 700000 HCMC, Viet Nam

A B S T R A C T

Introduction: Vitamin D₃ plays a vital role in bone health, with low levels of vitamin D₃ being related to skeletal fragility, fractures, and metabolic disorders such as diabetes. Metformin is known as an anti-hyperglycemic agent for regulating blood sugar. A correlation between diabetes mellitus and osteoporosis is attracting considerable interest, and research to find the prevention and treatment is gradually being studied. In this study, we investigated the effect of metformin and vitamin D₃ on osteogenic differentiation of human adipose tissue-derived mesenchymal stem cells (AT-MSCs) under high D-glucose concentrations and optimized by combining vitamin D₃ and metformin in the process.
Methods: ROS production of AT-MSCs under high D-glucose conditions was measured by DCFH-DA assay. The differentiated AT-MSCs were analyzed by Alizarin Red S staining and optical density measurement. The investigation involved the examination of osteogenic master genes' expressions using quantitative reverse transcription polymerase chain reaction (qRT-PCR) techniques.
Results: Interestingly, the results have shown that human AT-MSCs will exhibit high ROS accumulation and low osteogenic differentiation capabilities, indicated by low calcium deposition, as well as low expression of indicative genes such as *ALP, Runx-2* under high D-glucose conditions. The combination of vitamin D₃ and metformin remarkably accelerated the osteogenic differentiation of AT-MSCs under high D-glucose concentrations more effectively than the administration of either agent.
Conclusions: This study partially explains an aspect of an *in vitro* model for pre-clinical drug screening for osteoporosis-related diabetic pathological mechanisms, which can be applied for further research on the prevention or treatment of osteoporosis in diabetic patients.
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1. Introduction

Diabetes mellitus (DM) is distinguished by hyperglycemia, elevated levels of blood sugar, stemming from the dysfunction of insulin production, insulin action, or both. Patients with DM are associated with higher risks of lowered bone density due to the various complications caused by the disease [1,2], leading to increased risk of bone fracture. A common condition of DM is increased reactive oxygen species (ROS) formation elevated receptor activator of nuclear factor kappa beta (NF-κB) ligand and receptor for advanced glycation endproducts expression, which is expressed in osteoclasts and stimulates osteoclast productions, adding to the reduction of bone mass [3–5]. Previous reports demonstrated that ROS production also increases in mesenchymal stem cells-derived from diabetic donors [3,4].

Metformin is a first-line medication for the treatment of type 2 diabetes and has been shown to have benefactor potential for bone health of diabetic patients. The mechanism action of

* Corresponding author. School of Biomedical Engineering, International University, 700000 HCMC, Viet Nam.
** Corresponding author. School of Biomedical Engineering, International University, 700000 HCMC, Viet Nam.
E-mail addresses: vblong@hcmiu.edu.vn (L.B. Vong), tnthuy@hcmiu.edu.vn (N.-T. Trinh).

metformin on bone is not yet fully understood. However, an *in vivo* study hinted at the ability of metformin to improve glucose metabolism, insulin sensitivity, and to suppress inflammation via the regulation of AMPK, NF-κB, p65, and Hmgb1 gene expression [6]. In addition, metformin can diminish the negative effects of hyperglycemia on bone health [7]. It is worth noting that conflicting studies exist on the effect of metformin on bone health. A recently published meta-analysis indicated that there is not strong correlation between decreased fracture risk and metformin administration. However, the study was conducted with limited data samples [8]. Further investigations about metformin in bone health are necessary to provide a concrete conclusion. On the other hand, metformin induces osteoblastic differentiation of human dental pulp stem cells and induced pluripotent stem cell-derived mesenchymal stem cells [9,10].

On the other hand, vitamin D_3 (1,25-dihydroxyvitamin D3 $(1\alpha,25(OH)_2D_3)$, an active form of vitamin D, is one of the primary factors regulating the osteoblast differentiation [11], which mainly works by attaching to its nuclear vitamin D receptor (VDR) [12,13]. According to previous reports, vitamin D_3 and VDR are crucial for the control of osteogenic differentiation of stem/progenitor cells [14—17]. Studies have revealed that vitamin D_3 stimulates the calcification of human osteoblasts and induces the osteogenesis of human MSCs [18,19]. During the process of bone development, *Runx-2* and *ALP* are the master transcription factor that is necessary for determining the cell lineage of osteoblasts [20].

Vitamin D_3 and metformin both have been shown to have positive effects on skeletal disorders and insulin resistance in previous studies [21,22]. Vitamin D_3 has been studied nearly entirely in terms of its function in the calcium homeostasis [23]. At the same time, metformin is a commonly used anti-hyperglycemic medication that reduces hepatic insulin resistance by acting as an insulin sensitizer [24]. Many studies have shown that metformin induced bone formation in diabetic patients [25] and diabetic mice models [26]. Moreover, the present results also demonstrated that osteogenic differentiation ofAT-MSCs was improved by the addition of vitamin D_3, but there were few studies about the effect of vitamin D under high D-glucose levels on the osteogenesis of MSCs. On the other hand, the effect of high D-glucose levels, vitamin D_3, metformin, and the combination of both vitamin D_3 and metformin on the properties of AT-MSCs, including proliferation, differentiation, migration, and toxicity should be examined to complete the stimulation of osteoporosis-related diabetes stem cell model, which is further employed in pre-clinical drug screening and osteoporosis-related diabetes researches, such as treatment and prevention.

In addition, our previous studies showed that insulin resistance-related gene induction was significantly elevated while the osteogenic ability in human AT-MSCs was impaired under 25 mM, 50 mM, and 100 mM D-glucose treatment [27,28]. Hence, we hypothesize that AT-MSCs may be a good candidate for examining the effect of vitamin D_3 and metformin on the osteogenic differentiation under high D-glucose conditions. Therefore, we aim to investigate the optimal dosages for the combination of metformin and vitamin D_3 treatment in human AT-MSCs under high D-glucose conditions.

2. Materials and methods

2.1. Stem cell culture

Human adipose tissue-derived mesenchymal stem cells (AT-MSCs) were provided by the Laboratory of Stem Cell Biology and Regenerative Medicine, University of Tsukuba, Japan. The ethical declaration were mentioned in our previous reports [29,30]. AT-MSCs were cultured in complex Iscove's modified Dulbecco's medium (IMDM, Gibco, USA), with 10 % fetal bovine serum (FBS, Gibco, USA), 1 % Pen-strep (Gibco, Sigma), and 5 ng/ml human bFGF (Sigma, USA) at 37 °C, 5 % CO2 and 98 % humidity. The fresh medium was replaced every three days. Cells were stocked in solution including 90 % FBS and 10 % dimethylsulfoxide (DMSO, Sigma, USA) at −80 °C or liquid nitrogen. The AT-MSCs that were used for all experiments were in passages 9—12.

2.2. The supplement of high D-glucose concentrations

The D-glucose solutions at different concentrations (25, 50, and 100 mM) (Sigma, USA) were supplemented to the osteogenic induced-medium before adding to the culture plates used for investigating ROS release and osteogenic differentiation process.

2.3. DCFH-DA assay

AT-MSCs were seeded in 24-well-plate and incubated at 37 °C, 5 % CO_2 overnight. Hydrogen peroxide (H_2O_2) (2 mM) diluting in serum-free medium was used as the positive control sample. High D-glucose (25, 50 and 100 mM) was supplemented to the plate and cultured for three days. DCFH-DA (40 μM) was added and incubated for 30 min to interact with the ROS inside the cells. Images were taken using a fluorescence microscope (Nikon, Japan) at magnification of 10×. The fluorescent signal was measured using microplate reader (Thermo, USA) at 495 nm and 535 nm for excitation and emission, respectively.

2.4. In vitro osteogenic differentiation

The osteogenic induction in AT-MSCs was referenced from a previous study [29]. AT-MSCs were seeded in 48-well-plate with the density of 3×10^4 cells/well and cultured at 37 °C, 5 % CO_2, and 98 % humidity for 24 h. The osteogenic differentiation medium was made of IMDM, 1 % FBS, 1 % antibiotics, supplemented with dexamethasone (Sigma, USA), human epidermal growth factor (hEGF, Sigma, USA), vitamin C (Sigma, USA), and β-glycerol-2-phosphate (β-GDP, Sigma, USA). The complex medium was added to the cells the day after seeding. The medium was freshly renewed every 3 days in total 25 days. The change in cell morphology was captured by using NIS-Elements BR software at day 0 (before differentiation) and day 25 in order to express changing of AT-MSCs to differentiated cells.

2.5. Alizarin Red S staining for osteogenic differentiation recognition

Calcium formation of AT-MSCs was qualified by the Alizarin Red staining method [29] at day 25. Differentiated cells were fixed with formalin 10 % for 10 min and rinsed with distilled water. Alizarin Red S (Sigma, USA) was added to the cells and incubated for 5 min. Images were captured under an inverted microscope (Nikon, Japan) at magnification 10×. Then cell lysis buffer was added to measure optical density at 480 nm by using microplate reader (Thermo, USA).

2.6. Total mRNA isolation

AT-MSCs were seeded in a 24-well-plate with the density of 5×10^4 cells/well and underwent osteogenic differentiation process. mRNA was isolated using Sepasol-RNA I Super G (Nacalai tesque, Japan) on day 7 of the process.

Table 1
The primers employed for quantitative polymerase chain reaction.

Function	Gene	Primer	Sequence
Internal control	*β-actin*	5′-primer	GTGCGTGACATTAAGGAGAAGCTGTGC
		3′-primer	GTACTTGCGCTCAGGAGGAGCAATGAT
Osteogenic markers	*Runx-2*	3′-primer	CAGATGGGACTGTGGTTACTGTCATGG
		5′-primer	CCTAAATCACTGAGGCGGTCAGAGAAC
	ALP	3′-primer	ACGTGGCTAAGAATGTCATC
		5′-primer	CTGGTAGGCGATGTCCTTA

2.7. Reverse transcriptase polymerase chain reaction

Total RNA was firstly reverse-transcribed by High-Capacity cDNA Reverse Transcription Kit (Thermo, USA). Complementary DNA (cDNA) was used to examine the expression of *Runx-2*, and *ALP* by real-time PCR (q-PCR) using Maxima SYBR Green/ROX qPCR Master Mix (2X) Kit (Thermo, USA) and reacted in Qiagen Rotor-Gene Q. Expression of genes in interest were then calculated by the $2^{(-\Delta\Delta Ct)}$ formula. The internal control utilized in the study was β-actin. Table 1 displays the primers employed in the PCR experiments [28,29].

2.8. Statistical analysis

One-way analysis of variance (ANOVA) (Tukey post-hoc test; SPSS 20 software, IBM Corp.) was used to identify significant differences between groups. The experiments conducted in the study were replicated by a minimum of 3 times, with a significance level of p < 0.05 being accepted as statistically significant. The data were presented as the mean standard deviation (SD).

3. Results

3.1. High D-glucose concentrations induced ROS generation

The internal ROS generation of AT-MSCs under different conditions, including normal and high D-glucose concentrations (25, 50, and 100 mM), was investigated by DCFH-DA assay. The result showed that high D-glucose at 25, 50, and 100 mM had increased the internal ROS release in AT-MSCs (Fig. 1A). Furthermore, the intensity of ROS in 25 mM and 50 mM-treated groups was not significantly different. Remarkably, the ROS production under 100 mM D-glucose has increased 2.35-fold ($p < 0.005$, $n = 3$) and 1.61-fold ($p < 0.005$, $n = 3$) higher than that in the control group and 25 mM D-glucose-treated group, respectively (Fig. 1B). The results indicated that the characteristic of AT-MSCs, such as osteogenic differentiation under high D-glucose conditions, may be altered due to the over-expression of ROS.

3.2. The effect of metformin concentrations on the osteogenic differentiation ability of AT-MSCs under high D-glucose conditions

In this study, two concentrations of metformin (25 and 50 μM) were added to the osteogenic differentiation-induced medium. Under high D-glucose concentration (100 mM), the osteogenic differentiation ability of AT-MSCs was inhibited (Fig. 2A). The optical density (OD) measurement of 100-mM D-glucose treated group was significantly lower than the untreated group (2.05-fold lower, $p < 0.05$, $n = 3$) (Fig. 2B). Moreover, it was also indicated by the reduction in the expression of osteogenic-specific gene *Runx-2*. The expression of *Runx-2* in the 100-mM treated group was 1.75-fold ($p < 0.05$, $n = 3$) lower than the untreated group (Fig. 2C). Overall, treating with metformin (25 and 50 μM) has not shown any supportive effect on osteogenic differentiation of AT-MSCs under high D-glucose conditions. Under high D-glucose conditions, the calcium deposition in 50-μM treated groups was significantly lower
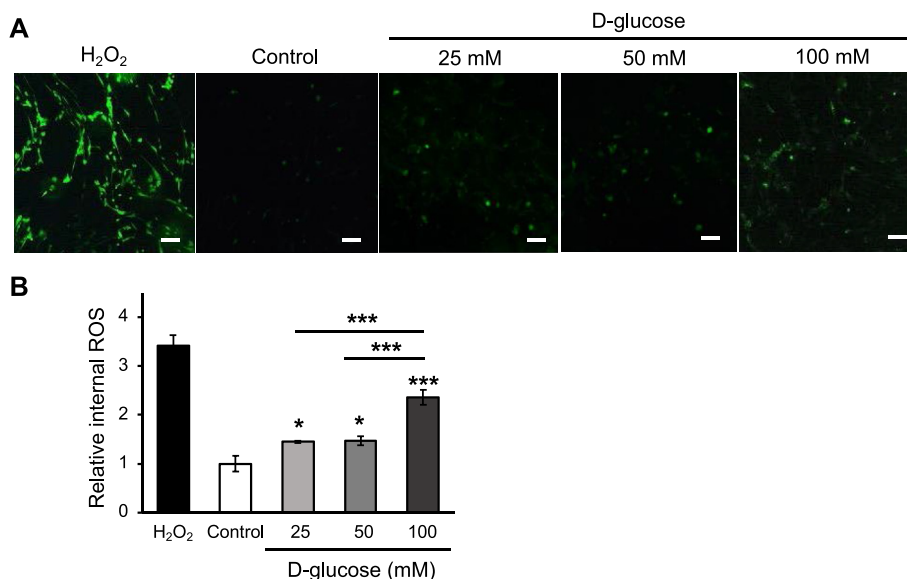

Fig. 1. The ROS generation of AT-MSCs under 25, 50, and 100 mM D-glucose conditions were recognized by DCFH-DA assay. (**A**) The fluorescent-stained cells were captured under a fluorescent microscope. Control: non-treated AT-MSCs. The scale bar is at 100 μm; (**B**) The histogram indicated ROS release of AT-MSCs under different conditions. The data are expressed as the means ± SD, ***$p < 0.001$, *$p < 0.05$, $n = 3$.
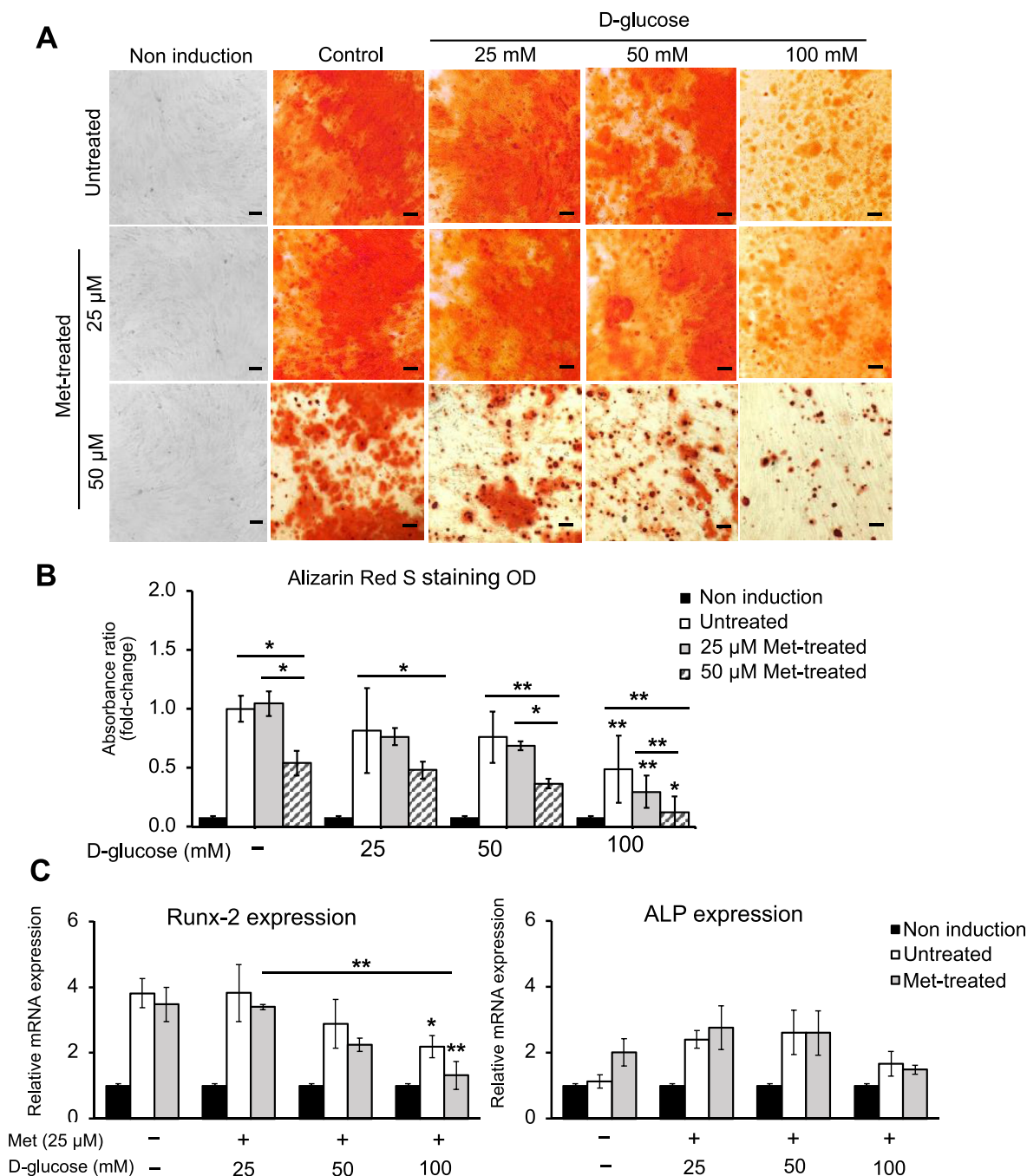
Fig. 2. The effect of different concentrations of metformin (25 and 50 μM) on osteogenic differentiation of AT-MSCs under high D-glucose concentrations (25, 50, and 100 mM). (**A**) The matrix mineralization of AT-MSCs was recognized by Alizarin Red S (stained in orange red) at day 25 of osteogenic differentiation process. The scale bar is at 100 μm. (**B**) The quantitative result of calcification of AT-MSCs was measure at wavelength 480 nm. (**C**) The effect of metformin (25 μM) on the expression of osteocyte-specific genes *Runx-2* and *ALP* under high D-glucose conditions. The level of mRNA expression was measured by qRT-PCR on day 7 of osteogenic induction and normalized to β-actin. The data demonstrated the average values of three independent experiments (mean ± SD); $n = 3$. *$p < 0.05$, **$p < 0.01$. Non-induction: the undifferentiated group; Untreated: the differentiated group without being treated with either metformin or D-glucose.

than those in the untreated groups (Fig. 2B). However, the osteogenic differentiation under 25 μM metformin had no significant difference compared to the untreated group (Fig. 2B). Moreover, the expression of *Runx-2* and *ALP* under metformin (25 μM) treated groups was similar to those in the untreated (Fig. 2C). This indicated that 50 μM metformin was inappropriate to support osteogenic differentiation of AT-MSCs under high D-glucose conditions. Hence, we suggested using 25 μM metformin for further experiments.

3.3. Evaluation of vitamin D₃ concentrations on osteogenic differentiation

We evaluated the different concentrations of vitamin D₃ (1, 2, and 10 nM) on osteogenic differentiation. The staining result demonstrated that vitamin D₃ at 1 nM has increased the calcium deposition significantly compared to the other groups (Fig. 3A). The OD measurement supported that the calcium accumulation in 1 nM
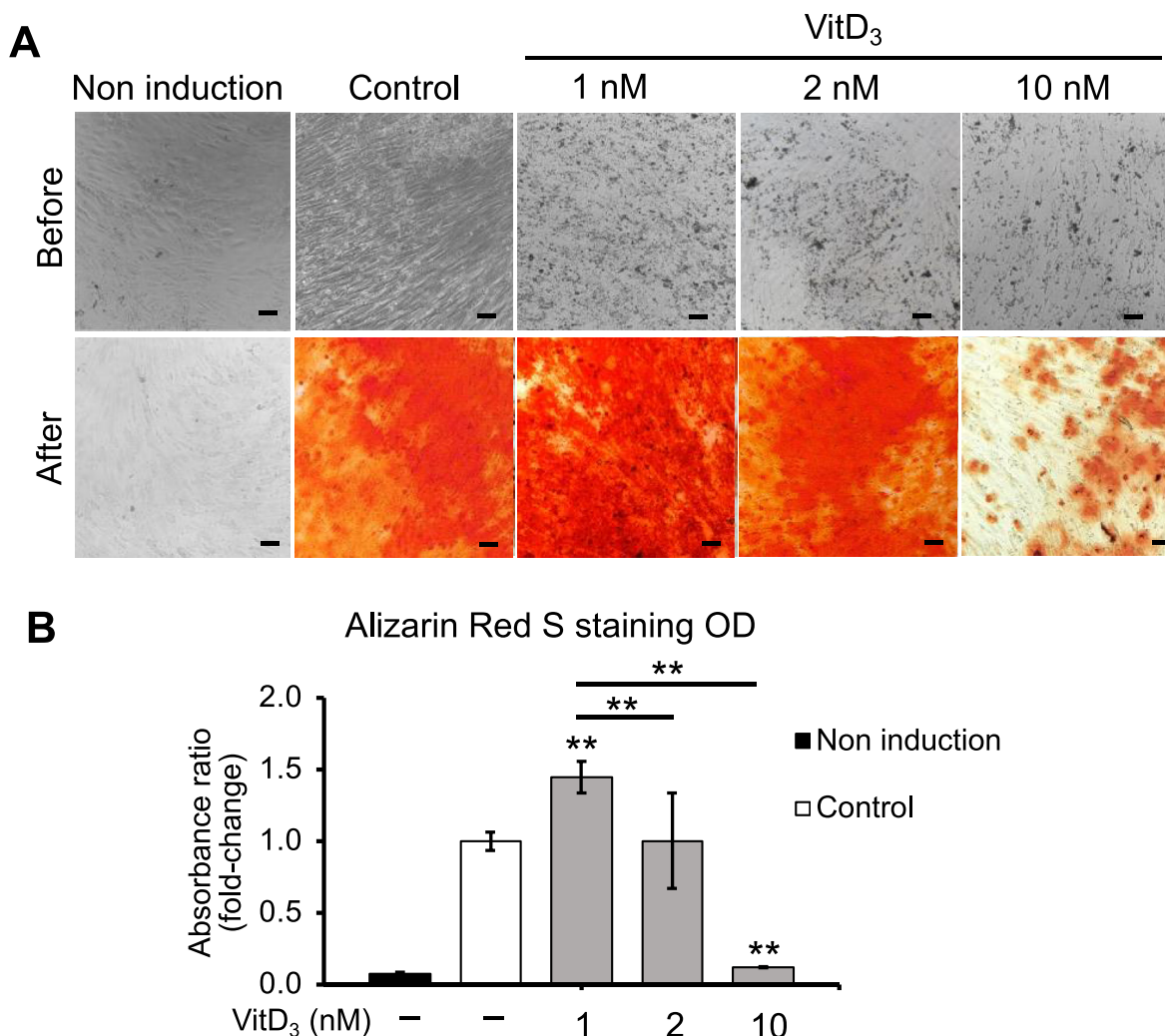
Fig. 3. The effect of vitamin D_3 on osteogenic differentiation ability of AT-MSCs. (**A**) Qualitative result of differentiated cells under different concentrations of vitamin D_3 (1, 2, and 10 nM). The scale bar is at 100 μm. (**B**) The quantification result of AT-MSCs supplemented with vitamin D_3 (1, 2, and 10 nM). The data demonstrated the average values of three independent experiments (mean ± SD); $n = 3$. **$p < 0.01$.

treated group was increased 1.45-fold higher ($p < 0.01$, $n = 3$) than that in the untreated groups (Fig. 3B). Moreover, when increasing the concentration of vitamin D_3 to 2 nM, we observed that the ability to differentiate to osteoblasts of the untreated group and 2 nM treated group were similar (Fig. 3B). Surprisingly, the pre-stained result of 10 nM vitamin D_3 showed detachment of cells during the differentiation process (Fig. 3A), as well as the inhabitation in osteogenic differentiation (Fig. 3B). The Alizarin Red S (ARS) staining of 10 nM treated group was 8.3-fold decreased ($p < 0.01$, $n = 3$) compared to the untreated group, indicating that this concentration of vitamin D_3 was not applicable for this study. Thus, we decided to use 1 nM and 2 nM of vitamin D_3 for further investigation.

3.4. Vitamin D_3 improved osteogenic differentiation under high D-glucose conditions

The effect of vitamin D_3 at those levels (1 nM and 2 nM) on osteogenic differentiation in the presence of varying amounts of high concentrations of D-glucose was investigated. Overall, when comparing the effect of two concentrations of vitamin D_3 (1 nM and 2 nM) on the enhancement of osteogenic differentiation under

various high D-glucose conditions, 1 nM of vitamin D_3 enhanced the mineralization of AT-MSCs more effectively (Fig. 4A). The quantification results showed that osteoblasts differentiated from AT-MSCs in 1 nM treated group was increased 1.87 ($p < 0.05$, $n = 3$), 1.72 ($p < 0.01$, $n = 3$), and 2.59 ($p < 0.05$, $n = 3$), compared to those in untreated groups under 25 mM, 50 mM, and 100 mM D-glucose, respectively (Fig. 4B). On the other hand, 2 nM of vitamin D_3 has inhibited the osteogenic differentiation under high D-glucose conditions instead of improving it (Fig. 4A). In correlation with staining results, the expression of *Runx-2* and *ALP* has been increased under the treatment of 1 nM vitamin D_3 (Fig. 4C). Although the presented data was not significantly comparable, the results showed the trend in upregulating osteogenic-specific genes using vitamin D_3 at 1 nM.

3.5. The investigation of synergistic effect of metformin and vitamin D_3 on osteogenic differentiation of AT-MSCs under different high D-glucose conditions

According to the previous results, metformin (25 μM) and vitamin D_3 (1 nM) have been selected to supplement examine for the synergistic effect on osteogenic differentiation under high D-glucose conditions. The staining result showed that the osteogenic
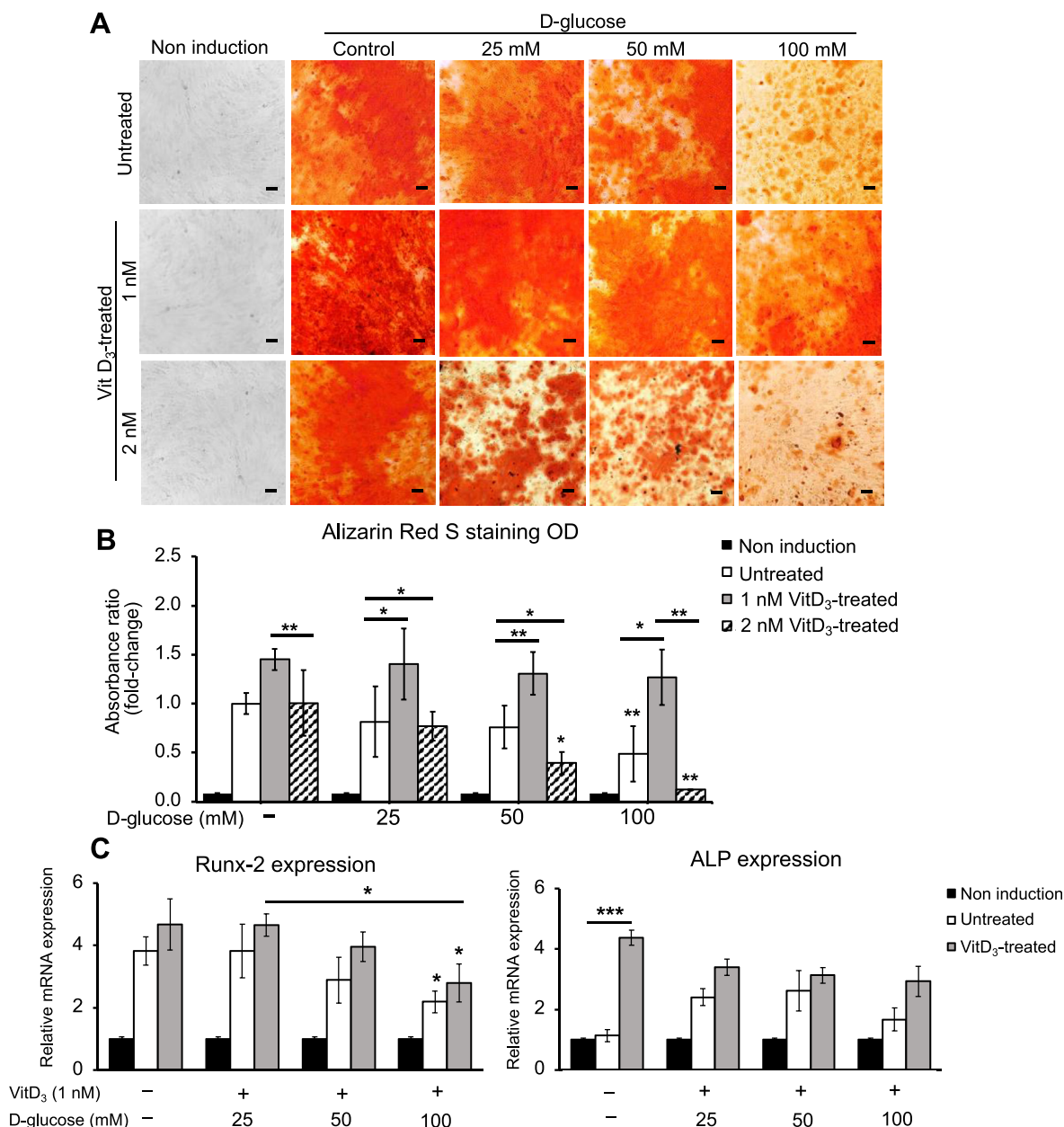
Fig. 4. The effect of vitamin D_3 (1 and 2 nM) on osteogenic differentiation ability of AT-MSCs under different concentrations of high D-glucose (25, 50, and 100 mM). (**A**) Qualitative result of differentiated cells under different high D-glucose concentrations supplemented with vitamin D_3 (1 nM and 2 nM). The scale bar is at 100 μm. (**B**) The quantification of calcium deposition of AT-MSCs. (**C**) The effect of vitamin D_3 (1 nM) on the expression of osteocyte-specific gene *Runx-2* and *ALP*. The data demonstrated the average values of three independent experiments (mean ± SD); $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

differentiation of AT-MSCs in the combined group (metformin+vitamin D_3-treated) was significantly increased compared to the untreated groups (Fig. 5A). The quantitative result of the combined group was 1.67-fold, 1.55-fold, and 2.22-fold ($p < 0.05$) higher than those of the untreated groups under 25 mM, 50 mM, and 100 mM D-glucose, respectively (Fig. 5B). Interestingly, osteogenic differentiation was progressively inhibited under 100 mM D-glucose condition, while the combined treatment of metformin and vitamin D_3 has shown their recovery effect. It was supported by the expression of *Runx-2* and *ALP* that was significantly enhanced in the combined group under high D-glucose condition (100 mM). *Runx-2* and *ALP* in the combined group have increased 1.82 ($p < 0.05$) and 6.94 ($p < 0.005$) times than the untreated groups (Fig. 5C). Moreover, we observed that combined groups showed better effects than

the single treatment with metformin or vitamin D_3. Although under 100 mM D-glucose condition, the increase in *Runx-2* expression of the combined group was not significant, the expression of *ALP* was 3.95-fold ($p < 0.005$) higher, compared to that in the vitamin D_3 treated group (Fig. 5D). The results indicated that the supplement of metformin has promoted the effect of vitamin D_3 in osteogenic differentiation under high D-glucose conditions, especially 100 mM.

4. Discussion

Although many studies have focused on bone marrow which is one of the main sources of mesenchymal osteoprogenitor cells, the success of bone-tissue regeneration depends on the osteogenic
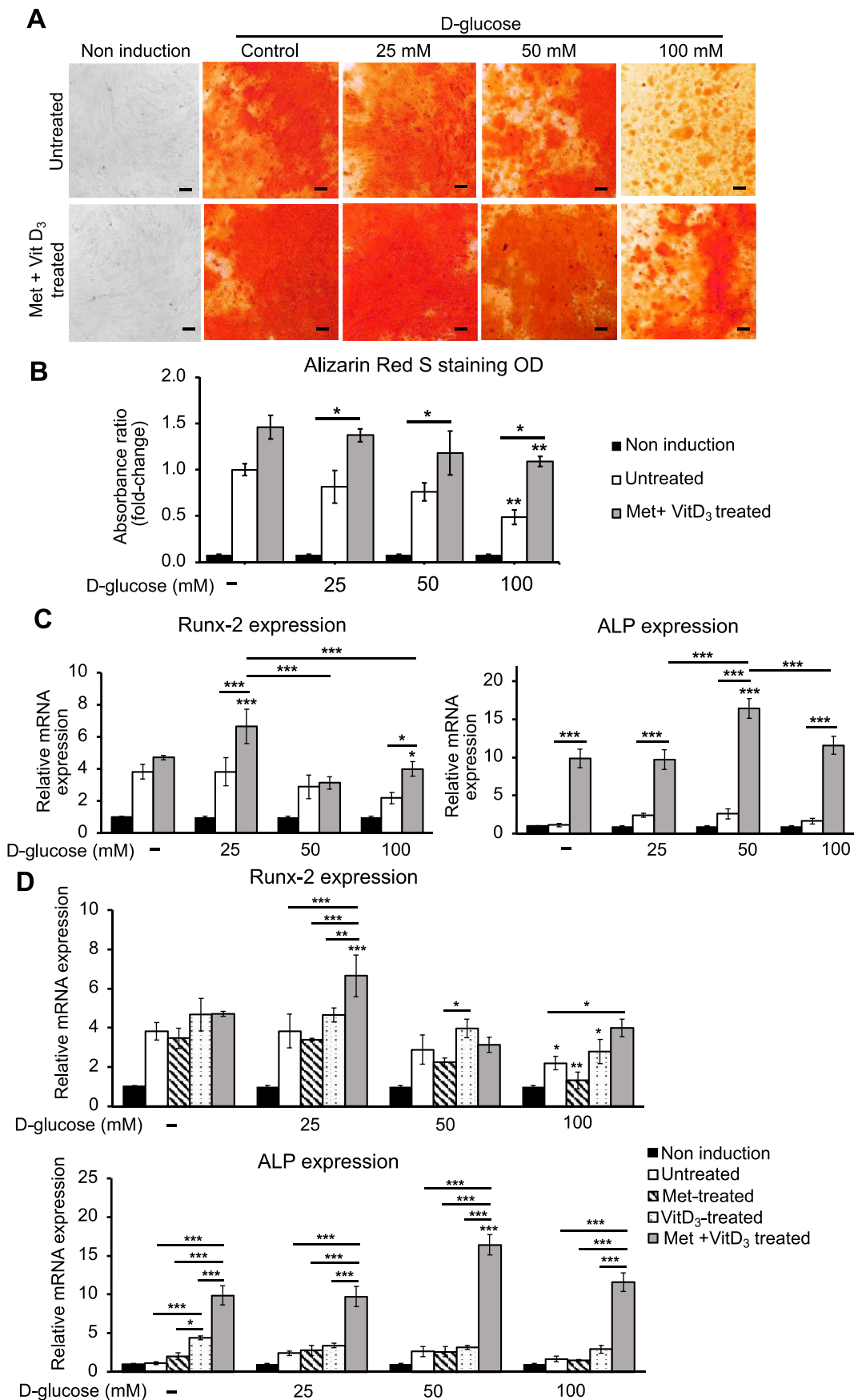
differentiation potential and secretomes. Both BM-MSCs and AT-MSCs have high osteogenic potential, osteoinductive ability, and immunomodulation properties. The secretomes of AT-MSCs has beneficial effects on recruiting other progenitor cells and can induce osteogenic differentiation *in vitro* and *in vivo* [31]. Osteoinduction is initiated by several transcription factors that govern AT-MSCs to an osteoblastic lineage. The key transcription factor commencing osteogenic differentiation of AT-MSCs is *Runx2*. After differentiation into preosteoblasts, Runx2, osterix, and β-catenin are in control of the cells to become immature osteoblasts. Then, *ALP* is a membrane-bound enzyme expressed as a marker in early differentiation and plays a key role in enhancing mineralization and calcification in these osteoblasts [32,33]. Notably, AT-MSCs has been proven to perform higher calcification and higher ability to regenerate new bone than BM-MSCs and dental pulp-derived MSCs in mouse bone fracture model. Besides, the advantage of AT-MSCs cells over BM-MSCs is the possibility to harvest them in a less invasive manner [31,34]. However, type 2 diabetic patients suffer from insulin resistance and chronic hyperglycemia, from which AT-MSCs' functions were impaired in osteogenic differentiation potential and wound healing ability [27,29,35]. In previous report, we also demonstrated that the expressions of insulin resistance-related genes *PTEN, EGR-1, GGPS-1* in healthy human AT-MSCs-treated with high ᴅ-glucose concentrations at 25 mM, 50 mM, and 100 mM were accelerated in comparison with those of human diabetic-derived AT-MSCs [27]. Additionally, according to our previous report, the expression of *ALP* and *Runx-2* were remarkably decreased in non-diabetic-derived AT-MSCs under high ᴅ-glucose conditions and in diabetic-derived AT-MSCs [28]. Hankamolsiri et al. (2016) also demonstrated high ᴅ-glucose concentration (25 mM) has downregulated the osteogenic genes in MSCs derived from other sources including the bone marrow, umbilical cord, placenta and chorion [36]. Therefore, in the scope of this study, we focused on a reverse effect of both metformin and vitamin D₃ on the osteogenic differentiation under high ᴅ-glucose concentrations at 25 mM, 50 mM, and 100 mM, which had obvious recovered not only the expression of *Runx2* and *ALP* which indicate the early osteogenic differentiation at day 7 of the process but also confirmed by Alizarin red S staining for localization and quantification of increasing calcium deposits at day 25 (Fig. 5). For futher investigation, we will investigate the molecular mechanisms of metformin and vitamin D₃ underlying the synergistic effects of the combination treatment on osteogenesis.

Here, we also provided more evidence about the effect of high ᴅ-glucose concentrations at 25 mM, 50 mM, and 100 mM on increasing intracellular ROS accumulation and inhibiting osteogenic differentiation in AT-MSCs, especially AT-MSCs-treated with 100 mM ᴅ-glucose (Figs. 1 and 2). Besides, we reported that metformin significantly reduced ROS release in AT-MSCs-treated with 100 mM ᴅ-glucose [37]. Our results are consistent with the report of Zhou R et al. (2020) and Marycz K et al. (2016) on mouse BM-MSCs and mouse AT-MSCs, respectively [38,39]. Besides, metformin at a concentration of 50 μM significantly enhanced the proliferation, migration, and osteogenic differentiation of periodontal ligament stem cells [40]. However, our result showed that metformin supplements at both 50 and 25 μM expressed little effect on improving suppressed osteogenic differentiation of AT-MSCs under high ᴅ-glucose concentrations (Fig. 2), suggesting that metformin alone

may not able to rescue the osteogenic differentiation potential under severe high glucose-induced oxidative stress.

Although we have not examined the effect of vitamin D₃ on ROS reduction in our research, vitamin D₃ has been proven to significantly reduce the intracellular ROS production in human AT-MSCs-treated with H₂O₂ [41]. Previous research has shown that vitamin D₃ at 10 and 50 nM has promoted osteogenic differentiation of iPS-derived osteoprogenitor cells [42]. However, our results indicated that 10 nM vitamin D₃ has not supported AT-MSCs osteogenic differentiation and caused cell detachment leading to a decrease in mineralization (Fig. 3A). This was consistent with the result of Mostafa et al. [43], vitamin D₃ (10 nM) combined with Dex in osteogenic induced-medium caused a reduction in cell proliferation and mineralization in hBM-MSCs. On the other hand, we investigated the smaller concentrations of vitamin D₃ (1 and 2 nM), and the results demonstrated that 1 nM of vitamin D₃ has increased osteogenesis under high ᴅ-glucose conditions (Fig. 4). It was similar to the previous study, vitamin D₃ (1 nM) has shown its effect in promoting dexamethasone (Dex), resulting in increasing *ALP* activity and matrix mineral formation [15]. Although the staining results of vitamin D₃ treated groups showed a significant increase in matrix mineralization (Fig. 4B), the expression of *Runx-2* and *ALP* was only significantly enhanced under 100 mM ᴅ-glucose condition (Fig. 4C). This can be explained by the stage of differentiation that vitamin D₃ affected as it could enhanced not only early but also late-stage markers of osteogenic differentiation [15].

Additionally, previous research demonstrated that metformin had neither deleterious impact on the successful treatment of vitamin D deficiency in T2DM patients nor on the subgroup with osteoporosis [44]. Metformin and vitamin D₃ may have a synergistic effect on the heterogeneous structure of skeletal muscle, according to previous report [22]. Notably, we demonstrated that the inhibition of osteogenic differentiation in AT-MSCs according to the increase of high ᴅ-glucose conditions as well as the significant recovery in osteogenic differentiation of AT-MSCs treated with metformin (25 μM) and vitamin D₃ (1 nM), especially at 100 mM ᴅ-glucose (Fig. 5). Although the molecular mechanisms underlying the synergistic effects of the combination treatment of metformin and vitamin D3 on osteogenesis in human AT-MSCs has not been elucidated, metformin regulates osteogenesis by the AMPK-Runx2 signal pathway which increased *ALP* activity, collagen synthesis, osteocalcin production, and extracellular calcium deposition in AT-MSCs and BM-MSCs *in vitro* [38,39]. In addition, metformin directly enhances not only AMPK activation but also SIRT1 enzymatic activity to reduce oxidative stress. SIRT1/AMPK pathway also enforce the osteogenic differentiation potential of human MSCs under oxidative stress condition [45,46]. On the other hand, vitamin D₃ contributes to enhanced osteogenic differentiation of MSCs under oxidative stress condition via activating the endogenous antioxidant system [41]. Vitamin D₃ activates SIRT1-FOXO3 signaling pathways enforced the osteogenic differentiation potential of BM-MSC [47]. Furthermore, pre-administration of vitamin D₃ (10 nM or 20 nM) attenuates the ROS-induced damage and MSC dysfunction by activating the endogenous antioxidant system by SIRT1 signaling in bone remodeling regulation [47]. Taken together, the results of the present study suggest that metformin and vitamin D₃ exerts a protective role on osteogenic differentiation potential of AT-MSCs from relativelyhigh glucose-induced oxidative stress. For

Fig. 5. The combined effect of metformin and vitamin D₃ on osteogenic differentiation under high ᴅ-glucose conditions. (**A**) The qualitative results of differentiated cells in untreated and combined groups under high ᴅ-glucose conditions (25, 50, and 100 mM). The scale bar is at 100 μm. (**B**) The quantitative result of AT-MSCs supplemented with metformin (25 μM) and VitD₃ (1 nM), compared to the untreated groups. (**C**) The mRNA expression level of *Runx-2* and *ALP* in the combined groups. (**D**) The comparison in *Runx-2* and *ALP* expression among single treatment with Met, VitD₃, and combined treatment with Met and VitD₃. The data demonstrated the average values of three independent experiments (mean ± SD); *n* = 3. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

futher investigation, the regulatory mechanism of metformin and vitamin D_3 on the ROS-SIRT1-FOXO3 axis under high D-glucose conditions, and the effect of metformin and vitamin D_3 on bone formation in diabetic *in vivo* model should be elucidated, which will contribute in pre-clinical drug screening and osteoporosis-related diabetes applications.

5. Conclusions

This finding has demonstrated that the effect of a single treatment with metformin, vitamin D_3, and the combined treatment. The results indicated that high D-glucose conditions have negatively affected the osteogenic differentiation of AT-MSCs, especially at the concentration of 100 mM D-glucose. Metformin (25 μM) has not shown significant effect on promoting osteogenic differentiation under high D-glucose conditions. On the other hand, despite the fact that vitamin D_3 at 1 nM recover the ability to differentiate into osteoblasts of AT-MSCs under high D-glucose conditions, the changes at the molecular level were not highly evaluated. Interestingly, the combined metformin and vitamin D_3 treated groups have shown outstanding effects in recovering osteogenic differentiation of AT-MSCs under high D-glucose conditions, especially at 100 mM D-glucose. Our observations pave the way for future investigations to better explain the impact of vitamin D_3 and meformin on bone development under high glucose-stress conditions, such as investigating the timing of gene and protein expression during the process. The findings obtained from this study have the potential to enhance comprehension of the association between DM and osteoporosis, as well as advance the management of DM and its associated complications.

Author contributions

Conceptualization and methodology, N-T.T.; validation, investigation and data analysis, N.N-Y.H., H.T.K.T., P.N.U.P. N-T.T.; writing original draft preparation, N.N-Y.H., L.B.V., N-T.T.; supervision, L.B.V., N-T.T.; project administration, T-H.N., L.B.V., N-T.T.; funding acquisition, N-T.T. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors have no competing financial interests to declare.

Acknowledgments

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