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The impairment of osteogenic differentiation of human adipose tissue-derived mesenchymal stem cells under high D-glucose concentrations

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Abstract:

Type 2 diabetic patients have an increased risk of developing serious long-term complications such as cardiovascular disease, retinopathy, nephropathy, neuropathy, diabetic foot, and osteoporosis. However, the correlation between hyperglycaemia and osteoporosis has not yet been fully clarified. In this research, we investigated the effect of different high D-glucose concentrations (25, 50, and 100 mM) on osteogenic differentiation of human non-diabetic and diabetic adipose tissue-derived mesenchymal stem cells (nAT-MSCs and dAT-MSCs). The differentiated cells were qualified by Alizarin Red S staining and quantified by measuring the absorbance at 482 nm. The expressions of osteogenic master genes were examined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) methods. Interestingly, the osteogenic differentiation and the expression of osteogenic-specific genes (Runx-2 and ALP) decreased with an increase of D-glucose concentration. Our work contributes to the understanding of the relationship between hyperglycaemia and osteoporosis and support the role of stem cells in developing new medicine or therapeutic treatment for preventing diabetic complications.

Keywords: **AT-MSCs, diabetes, differentiation potential, high D-glucose concentrations, osteoporosis.**

Classification number: **3.6**

Introduction

Diabetes mellitus (DM) brings a significant national and global disease burden that, according to the WHO (World Health Organization), is expected to affect the lives of 380 million people by the year 2025. Type 2 DM is found in up to 95% of people with DM [1]. According to previous research, chronic hyperglycaemia not only damages the organs but also had a deleterious effect on the skeletal system, degeneration of bone quality, loss of bone strength, increased fracture risk, and delayed bone healing [2]. Osteoporosis is a condition in which the density of osteocytes in the bones fall over time causing the bones to become increasingly brittle and prone to injury or fracture even under minor force. Despite the fact that DM and osteoporosis are two different diseases, there is a lot of evidence and speculation that the two diseases are genetically linked [3]. With an increasing proportion of patients with DM having a risk of bone fractures, the exact impact on bone structure is complex and has been poorly investigated [4, 5]. Mesenchymal stem cells, which have been widely known for their self-renewable, differentiation potential, and immunosuppressive properties, has demonstrated therapeutic effects in the treatment of osteoporosis in preclinical animal models [6, 7]. Adipose tissue-derived mesenchymal stem cells (AT-MSCs) have been shown to be abundant sources that have many outstanding properties for stem cell therapy compared to other source-derived stem cells.

According to Y.M. Li, et al. (2007) [8], AT-MSCs have shown the ability to differentiate into osteocytes under 25 mM D-glucose. Consistent with previous research, high D-glucose (25 mM) was proven to promote

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osteogenic differentiation in periodontal ligament stem cells (HDLSCs) [9]. However, persistent high glucose concentrations and AT-MSCs from diabetic donors with T2DM altered the osteogenic differentiation potential of AT-MSCs, which was demonstrated by ALP gene expression under 5.5, 13.5, and 55.5 mM D-glucose [10]. Moreover, high D-glucose concentration has been shown to suppress the expression of ALP and osteogenic gene Runx-2 [11-13]. ALP is an early marker of osteoblast differentiation, and its increased expression is associated with the progressive differentiation of osteoblasts [14]. Besides, Runx-2 is a critical regulator during osteogenic development, and the loss of Runx-2 expression at this early stage impairs osteogenic differentiation in bone development [15]. According to W. Xia, et al. (2022) [16], Runx-2 expression was decreased under the treated of 25 mM D-glucose in MC3T3-E1 cells. On the other hand, we recently identified that the AT-MSCs under high D-glucose concentrations (25, 50, and 100 mM) induced the expression of EGR-1, PTEN, and GGPS-1, which are involved in insulin resistance [17]. All of these findings contributed to the clarification of the relationship between T2DM and osteoporosis, as well as the improvement of stem cell therapy for the diseases. However, there are very few studies and a lot of controversy over the effect of the high D-glucose on osteogenic differentiation. Therefore, the aim of this study is to demonstrate the quantification and qualification of osteogenic differentiation of AT-MSCs from type 2 diabetic donors and non-diabetic donors under high D-glucose concentrations.

Materials and methods

Stem cell culture

Non-diabetic AT-MSCs (nAT-MSCs) and diabetic AT-MSCs (dAT-MSCs) were provided by the Laboratory of Regenerative Medicine and Stem Cell Biology, University of Tsukuba, Japan. These cells were characterized in previous reports [18, 19]. nAT-MSCs and dAT-MSCs were cultured in Iscove's modified Dulbecco's medium (Thermo, USA), supplemented with 10% foetal bovine serum (Thermo, USA), 1% antibiotics (Sigma, USA), and 5 ng/ml basic fibroblast growth factor (bFGF, Sigma, USA) at 37°C and 5% CO_2. The medium was renewed every 3 days until reaching 70-80% confluence, then cell passages were performed. nAT-MSCs and dAT-MSCs were cryopreserved in cell banker solution (Sigma, USA) and stored in liquid nitrogen for further experiments. All AT-MSCs used for this study were at passage 5-8.

Cell proliferation assay

nAT-MSCs and dAT-MSCs were seeded in a 24-well plate at density of $1.5x10^4$ cells/well and supplemented with high D-glucose concentrations (25, 50, and 100 mM). The cell culture medium was replaced every 3 days. The proliferation rate of AT-MSCs was investigated for 11 days after supplement with D-glucose. Trypan blue exclusion method was used to determine cell proliferation for every 48 hours until 11 days.

In vitro osteogenic differentiation of AT-MSCs

nAT-MSCs and dAT-MSCs were cultured in culture medium until reaching 100% confluence, then the cells were changed into a new medium with or without the induced osteogenic differentiation medium (including dexamethasone, β-glycerol-2-phosphate, ascorbic acid, human epidermal growth factor) [20]. The nAT-MSCs and dAT-MSCs, which were used as negative controls in the experiment, were cultured in normal medium.

Osteogenic differentiation qualification and quantification

After 21 days of the induction of osteogenic differentiation, the cells were fixed by 10% formalin (Merck, Germany) and the differentiation cells were qualified by Alizarin red S (Sigma, USA) staining to examine calcification in osteoblasts. The osteoblasts were visualized in red under an inverted microscope at 10x magnification. After that, osteoblasts were qualified and measured at 482 nm using a 96-well-plate microplate reader.

Quantitative reverse transcription polymerase chain reaction

To examine the expression of genes related to osteogenic differentiation, nAT-MSCs and dAT-MSCs were assessed at day 7 after differentiation induction. RNA was extracted using Sepasol-RNA I Super G (Nacalai Tesque, Japan). Total RNA (300 ng) was reverse-transcribed using reverse transcription polymerase chain reaction (RT-PCR) Kit (TOYOBO, Japan) to create a cDNA library. cDNA was analysed using a LightCycler 96 System (Roche, Switzerland) using Maxima SYBR Green/ROX qPCR Master Mix (2X) Kit (Thermo, USA). The expression levels of the target genes were analysed using the method. The β-actin gene was used as an internal control for the experiments. The sequences of the primer sets used for the PCR reactions are shown in Table 1.

Table 1. Primers used for quantitative polymerase chain reaction.

Function	Gene	Primer	Sequence
Internal control	β-actin	5'-primer	GTGCGTGACATTAAGGAGAA GCTGTGC
		3'-primer	GTACTTGCGCTCAGGAGGAG CAATGAT
Osteogenic markers	Runx-2	3'-primer	CAGATGGGACTGTGGTTACT GTCATGG
		5'-primer	CCTAAATCACTGAGGCGGTC AGAGAAC
	ALP	3'-primer	ACGTGGCTAAGAATGTCATC
		5'-primer	CTGGTAGGCGATGTCCTTA

Statistical analysis

Significant differences among various groups were performed using student's t-test and one-way analysis of variance (ANOVA) (Tukey post-hoc test; SPSS 20 software, IBM Corp.). The experiments in the research were repeated at least 3 times, and the $p<0.05$ value was considered as a statistically significant difference. Data was presented as the mean ±standard deviation (SD).

Results

The impairment of nAT-MSCs under high D-glucose concentrations

Glucose is considered the energy source of cells. However, high D-glucose concentrations can induce the senescence of cells via promoting ROS production [21, 22]. Thus, we evaluated the effect of different D-glucose concentrations (25, 50 and 100 mM) on the proliferation of nAT-MSCs for 11 days and compared them with the dAT-MSCs. The doubling time result indicated that there was no significant difference in the growth rate between nAT-MSCs (35.67±0.49 h) and dAT-MSCs (34.9±1.26 h). On the other hand, the high D-glucose supplemented groups showed a lower proliferation rate than the control group and dAT-MSCs (Fig. 1). Significantly, we found that the doubling time of the highest D-glucose treated group was 1.70-fold decreased (60.95±4.18 h, $p<0.01$, n=3), compared to the control group. This result proves that high D-glucose concentrations decelerated the growth of nAT-MSCs. However, the high D-glucose treated cells continued to proliferate, which is considered to be suitable for further experiments.

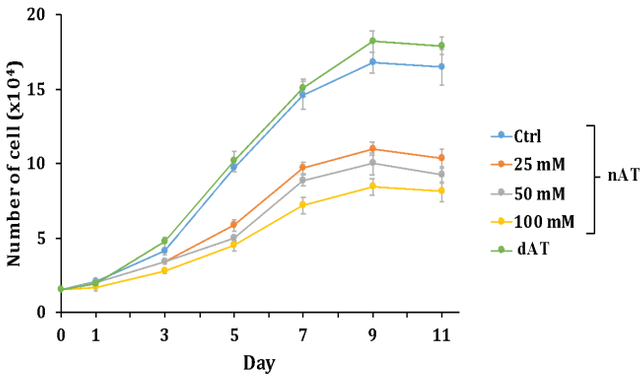

Fig. 1. The proliferation curve of nAT-MSCs under the effect of high D-glucose concentrations, compared with dAT-MSCs. The number of cells were determined every 48 hours by using trypan blue exclusion method for 11 days after D-glucose supplementation. High D-glucose concentrations inhibit the proliferation of nAT-MSCs. The data indicated the average values of three independent experiments (mean ± SD); Ctrl: control group is the non-treated group.

Qualification of the osteogenic differentiation in nAT-MSCs under high D-glucose concentrations

We hypothesized that the high D-glucose treated nAT-MSCs had decreased osteogenic differentiation according to the concentrations. To test this hypothesis, the osteogenic potential of nAT-MSCs was examined under high D-glucose concentrations (25, 50, and 100 mM). After 21 days, the osteoblasts were stained with Alizarin Red S, which is identified by the colour red (Fig. 2).

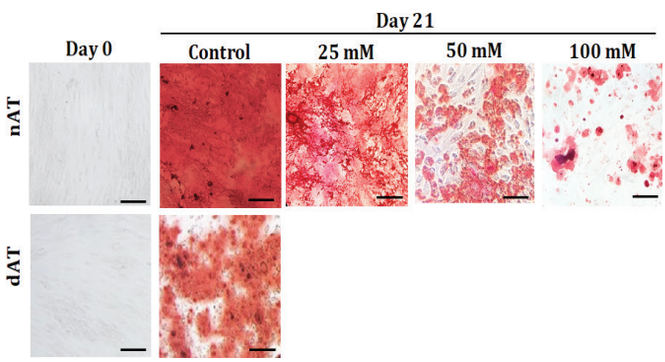

Fig. 2. The qualitative result of osteogenic differentiation of adipose tissue-derived mesenchymal stem cells (AT-MSCs) under the effect of high D-glucose concentrations. Osteoblasts were determined at day 21 using Alizarin red S staining, which indicated the calcification of the surface of osteoblasts (red). Scale bar: 50 μm.

The result demonstrated that osteoblasts differentiated from nAT-MSCs were greater than those from nAT-MSCs. On the other hand, osteoblasts differentiated from the high D-glucose-treated groups were worse than those

from the non-treated groups and dAT-MSCs (Fig. 2). The high D-glucose concentrations reduced the ability to differentiate into osteocytes of nAT-MSCs. This staining result suggested that high glucose conditions have a negative effect on osteogenic differentiation of mesenchymal stem cells.

Quantification of the osteogenic differentiation in nAT-MSCs under high D-glucose concentrations

To compare the significant difference in osteogenic differentiation of dAT-MSCs and nAT-MSCs under high D-glucose concentration, we quantified the osteoblasts that were differentiated from nAT-MSCs and dAT-MSCs by cell lysis and measured at 482 nm.

The result showed that the osteogenic differentiation ability of dAT-MSCs was significantly lower than that of non-treated nAT-MSCs ($p<0.05$, n=3 in each). The quantitative result showed that the calcification of nAT-MSCs in the high D-glucose-treated groups was significantly reduced as compared to the control group (25 mM, 1.16-fold ±0.04 decrease, $p<0.05$, n=3 in each; 50 mM, 1.77-fold ±0.05 decrease, $p<0.01$, n=3 in each; 100 mM, 7.4-fold ±0.08 decrease, $p<0.01$, n=3 in each) (Fig. 3). Interestingly, the level of osteogenic differentiation in dAT-MSCs was not significantly different between nAT-MSCs and dAT-MSCs. However, the increase in D-glucose concentration at 50 and 100 mM caused a remarkable decrease in osteogenic differentiation of nAT-MSCs compared to dAT-MSCs ($p<0.01$) (Fig. 3). Collectively, the higher D-glucose concentration the lower osteogenic differentiation.

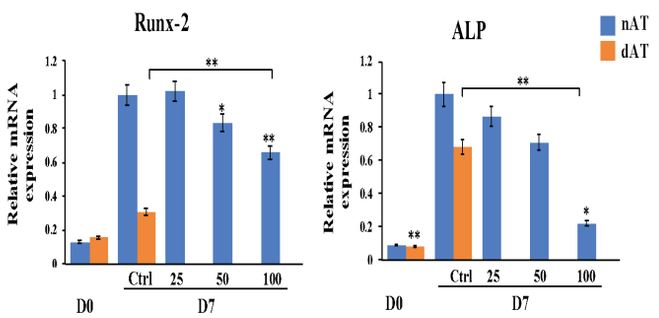

Fig. 3. The quantitative result of calcification of adipose tissue-derived mesenchymal stem cells (AT-MSCs) under different D-glucose concentrations. Absorbance was measured using microplate reader at 482 nm for Alizarin red S staining. The higher D-glucose concentrations were added, the lower absorbance ratio of Alizarin red S was determined. Data indicated average values of three independent experiments (mean ± SD); $p<0.05$ (*); $p<0.01$ (**); D0: day 0; D21: day 21.

High D-glucose concentrations decreased osteogenic gene expression

In agreement with the staining result, the expression of osteogenic-specific genes (Runx-2 and ALP) in AT-MSCs gradually decreased with high D-glucose concentrations compared with control groups (Fig. 4).

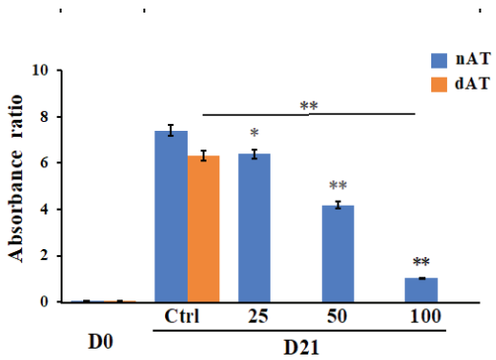

Fig. 4. The influence of high D-glucose concentrations on the expression of osteocyte-specific genes.

Runx-2 and ALP expression in the 25-mM D-glucose-treated group was not statistically different compared to nAT-MSCs in the control group. However, in the 50-mM D-glucose-treated group, Runx-2 expression decreased 1.20-fold ±0.06 ($p<0.05$, n=3 in each) and ALP expression decreased 1.41-fold ± 0.01 ($p<0.01$, n=3 in each) compared with nAT-MSCs in the control group. Besides, it was important to notice that the expression of Runx-2 in the 100-mM D-glucose-treated group was 2.13-fold±0.13 higher than in dAT-MSCs ($p<0.01$, n=3 in each), while the ALP expression was 3.1-fold ±0.47 lower than that in dAT-MSCs (Fig. 4). Therefore, these genes may play an important role in inhibiting the nAT-MSCs function under high D-glucose conditions in osteogenic differentiation, leading to impairing osteoblast production in diabetic conditions.

Discussion

AT-MSCs have been shown to have a great potential for therapeutic treatment [6]. However, AT-MSCs under diabetic condition (dAT-MSCs) had impaired functions of cell survival, migration, inflammation, and angiogenesis [19]. According to our previous report, nAT-MSCs have increased insulin resistance genes under high D-glucose concentrations (25, 50, and 100 mM) compared to dAT-MSCs [17]. In agreement with our previous study, these glucose concentrations inhibited cell proliferation and osteogenic differentiation of nAT-MSCs in comparison

of dAT-MSCs.

Persistent high D-glucose concentrations have decreased the growth rate of AT-MSCs at day 5 (Fig. 1). These findings were consistent with previous research that found high D-glucose concentration induced the senescence via an extrinsic caspase pathway [10, 23, 24]. However, we observed that at the concentration of 100 mM, nAT-MSCs were not completely gone in the apoptosis process. This indicated that 100 mM of D-glucose was considered as acceptable for further experiment, yet the threshold for nAT-MSCs.

In addition to the inhibition of the proliferation, we found that high D-glucose concentrations decreased the osteogenic differentiation of nAT-MSCs (Figs. 2-4). The results of qualification, quantification in osteogenic differentiation of nAT-MSCs under high D-glucose concentrations, and dAT-MSCs in cooperation with the expression of osteogenic-specific genes (Runx-2 and ALP). Runx-2 (Runt-related transcription factor 2) is the key factor that plays an essential role upstream of osteoblastic differentiation, which induces the expression of osteogenic extracellular matrix genes during osteoblast maturation such as collagen-I, alkaline phosphatase (ALP), and osteocalcin [15]. Besides, ALP (alkaline phosphatase) is an enzyme that is involved in the mineralization of bone. ALP is expressed early in development in both bone and calcifying cartilage tissues, and can be found on the cell surface and in matrix vesicles [14]. According to W. Hankamolsiri, et al. (2016) [24], despite the fact that they only investigated the effect of high D-glucose at 25 mM, they concluded that high D-glucose concentration did not affect osteogenic differentiation of both BM-MSCs and gestational tissue-derived MSCs. Another research has shown that high D-glucose at 44 mM decreased ALP activity and calcium deposition via increasing collagenase [25]. In this study, D-glucose at concentrations of 50 and 100 mM have shown remarkable inhibition in osteogenesis of nAT-MSCs based on the results of calcification and gene expression. These findings are consistent with clinical findings of the circumstance of diabetic patients having a higher chance of suffering from aplastic bone disease [26-28]. Although we found a negative effect of high glucose concentrations in nAT-MSCs in proliferation and osteogenic differentiation, further studies are necessary to determine a medication that can rescue the impaired osteogenic differentiation ability and its mechanism by manipulating gene expression in nAT-MSCs compared to dAT-MSCs.

Conclusions

This study demonstrated that high D-glucose concentrations not only inhibited the proliferation but also impaired the osteogenic differentiation of AT-MSCs by suppressing the expression of osteogenic-specific genes Runx-2 and ALP. The knowledge gained from this study contribute to the understanding of the relationship between T2DM and osteoporosis, suggesting the role of stem cells in developing new medicine or therapeutic treatment for preventing diabetic complications.

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COMPETING INTERESTS

The authors declare no conflicts of interest in association with the present study.

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