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OXIDATIVE STRESS RESPONSES OF IN VITRO EXPANDED WJ-MSCS DERIVED FROM MOTHERS WITH DIABETES MELLITUS

Aim. *Up to now, there are limited and conflicting data regarding the effects of diabetic microenvironment on perinatal stem cells. Our study aims to assess impact of diabetes mellitus on functional responses of in vitro expanded WJ-MSCs derived from DM-mothers to oxidative stress.* ***Methods.*** *MSCs were obtained by the explant method and cultured according to standard methods. Oxidative stress was induced by hydrogen peroxide (H_2O_2) added as a pulse. Treated WJ-MSCs were analyzed for metabolic activity by MTT assay.* ***Results.*** *The dose-response effect of different concentration of H_2O_2 (6,25–440 μM) on WJ-MSCs derived from mothers with DM and healthy mothers was evaluated in vitro. The difference between diabetic and control groups in NOAEL values (no observed adverse effect level) was analyzed.* ***Conclusions.*** *The effect of oxidative stress on the in vitro metabolic activity of both normal and diabetic WJ-MSCs was manifested according to the hormetic model of the response to the stressor (dose-response with stimulation by a low dose and inhibition by a high dose) and the threshold model with adaptation to low doses without stimulation. There was a trend towards higher tolerance to moderate oxidative stress in dWJ-MSCs compared with control cells according to NOAEL values. However, no statistically significant difference in these values between groups was found, according to the Mann-Whitney U-test. Further studies on more amount of samples are needed to use this cell model for diagnostic applications and to develop criteria for donor cells.*

Keywords: *WJ-MSCs, diabetic mellitus, oxidative stress.*

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Introduction

Diabetes mellitus (DM) and its complications significantly affect the quality of life and life expectancy of patients worldwide. According to The IDF Diabetes Atlas 2025, 589 million adults (29—79) are living with diabetes. This number is predicted to rise to 853 million by 2050 [https://diabetesatlas.org]. Traditional therapeutic drugs for DM have great limitations (they can only temporarily control blood glucose level, but not fundamentally restore it) and side effects. Currently, numerous studies have demonstrated the effectiveness of MSCs and their bioactive factors in managing DM [1—3]. However, in the last decade, it has been shown that the diabetic microenvironment can affect the therapeutic potential of MSCs, manifested by decreased cell proliferative activity, reduced chondrogenic and osteogenic differentiation potential, and increased levels of reactive oxygen species [4, 5]. It has been demonstrated that the diabetic microenvironment may reduce the therapeutic potential of both normal MSCs after transplantation into diabetic patients and donor cells obtained from diabetic individuals [6, 7].

Perinatal stem cells are highly attractive for cell therapy due to their unique features. The cells have promising immunomodulatory and regenerative properties and a number of advantages over adult stem cells. Currently, Wharton's jelly MSCs (WJ-MSCs) are widely studied due to their characteristics, abundance within the tissue and rapid proliferation. However, the pregnancy complications such as diabetes may affect extraembryonic cells properties, including WJ-MSCs, as evidenced by a number of studies [8—10].

Hyperglycemia and high glucose-induced oxidative stress are supposed to be the main factors in the diabetic complications [4, 11, 12]. At the same time, increasing number of studies indicate that MSCs possess antioxidant properties, which may explain their therapeutic effects in animal models of various diseases [13, 14]. According to antioxidant paradigm MSCs, "the presented studies evidently demonstrate that MSCs exhibit antioxidant potential either directly via scavenging of ROS and donating mitochondria or indirectly by upregulation antioxidant defenses in other cells and altering cellular bioenergetics" [13].Impact of diabetic environment under pregnancy on antioxidant efficacy of perinatal MSCs is still under investigation. Therefore, the aim of this work was to study the impact of the diabetic microenvironment on antioxidant potential of *in vitro* expanded WJ-MSCs derived from DM-mothers (dWJ-MSCs) and compare dWJ-MSCs and WJ-MSCs from healthy women (nWJ-MSCs) responses to oxidative stress. Specifically, our focus was on assessing the influence of DM environment on survival of WJ-MSCs *in vitro* under different levels of oxidative stress.

Methods

WJ-MSCs isolation

WJ-MSCs were isolated by non-enzymatic culturing of explants from human umbilical cords obtained from normal pregnancies (n = 3) and diabetic pregnancies (n = 3) as previously described [15]. In brief, the human umbilical cord tissue (hUC) was cut into 0.5—1 cm pieces, which were washed with culture medium and PBS, and the three vessels were removed carefully. These pieces were placed in 25 cm^2 flasks,containing DMEM/F-12, 10% FBS and cultured at 37 °C with 5% CO_2 under normoxic conditions (20% O_2). The cells expansion was monitored daily and passaged using 0.25% trypsin-EDTA when monolayer confluence was about 80—85%. The cells at second passage (P2) were used for study. To simulate oxidative stress conditions, cultures were exposed to typical membrane-permeable oxidant H_2O_2 from 6,25 to 450 μM for 24 hours.

MTT assay for determination of cell viability

The metabolic activity of WJ-MSCs was assessed using the MTT assay. Briefly, the cells (5×10^3 cells per well) were seeded on a 96-well plate. After 24 h, culture medium was removed and cells were

treated with H_2O_2 for 24 h.The control cells were cultured in DMEM/F12 medium without H_2O_2. After MTT treatment, the OD value was detected at 570 nm using a microplate reader [16]. Cell viability was expressed as a percentage of control.

Data analysis

Data are expressed as the mean ±SD. Dose response curves were plotted on Excel using the log scale for x-axis. Statistical analysis was performed using OriginPro 7.5 SR1 software (OriginLab Corporation, USA). The quantitative variables that followed a normal distribution were studied with analysis of variance (one-way ANOVA). Mann-Whitney U test was performed to compare quantitative variables not normally distributed. Box plots were created in the Microsoft 365 version of Excel. Statistical significance was considered as p < 0.05.

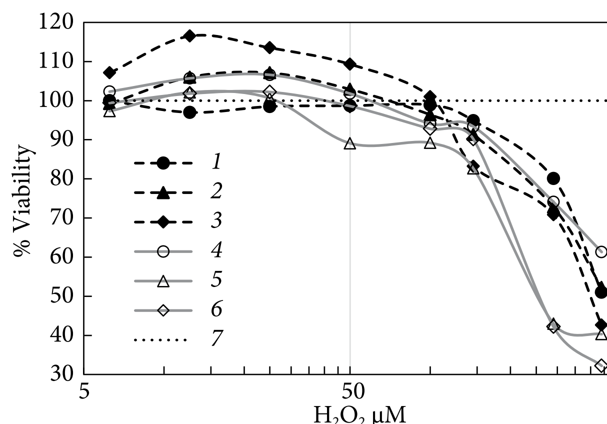

Fig. 1. Effect of H_2O_2-treatment on individual WJ-MSCs viability *in vitro*. *1* — dWJ-MSCs (T2); *2* — dWJ-MSCs (T2); *3* — dWJ-MSCs (T1); *4* — nWJ-MSC; *5* — nWJ-MSCs; *6* — nWJ-MSCs; *7* — the dotted line corresponds to 100% cell viability. The dashed lines correspond to dWJ-MSCs. A log scale was used for x-axis

Results and Discussion

Diabetes is known to be closely associated with hyperglycemia and oxidative stress. To estimate the impact of the diabetic microenvironment during pregnance on oxydative stress responses of *in vitro* expanded dWJ-MSCs, the cells isolated from healthy and DM individuals were cultured under difference oxidative stress levels and high glucose. According to WHO data normal blood glucose level in human usually range from 3,9 mM to 5,6 mM. We used DMEM/F12 culture medium, where the glucose content differs significantly from the physiological norm (17,5 mM). To simulate oxidative stress, the cells were exposed to single H_2O_2 treatment in the wide range of initial

Table 1. **Effect of diabetic microenvironment on WJ-MSCs performance under oxidative stress *in vitro***

µM	#1	#2	#3	#4	#5	#6
6,25	100.0 ± 2,5	99.2 ± 1	**107.1** ± 1*	102.3 ± 2.9	97.3 ± 2.5	99.4 ± 4.2
12,5	97.0 ± 3	**106.0** ± 1.1*	**116.5** ± 5.2*	**105.7** ± 1.9*	102.1 ± 2.6	101.8 ± 1.7
25	98.5 ± 3,9	**107.1** ± 2.1*	**113.5** ± 1.3*	**106.6** ± 2.5*	100.6 ± 2.3	102.2 ± 1.3
50	98.7 ± 3,5	102.9 ± 1.9	**109.3** ± 1.6*	101.8 ± 1.5	89.1 ± 4.1*	98.6 ± 3.1
100	98.9 ± 2,2	96.4 ± 2.5	101.0 ± 2.9	94.1 ± 2.4	89.2 ± 2.1*	92.8 ± 1.3*
145	94.9 ± 2,1	91.3 ± 3.2*	83.2 ± 2*	93.4 ± 2.6	82.7 ± 3.1*	90.2 ± 2.1*
290	80.1 ± 1,8*	72.8 ± 2*	70.8 ± 1*	74.1 ± 2.7*	41.3 ± 1.7*	42.2 ± 1.9*
440	51.0 ± 3,4*	52.2 ± 2.5*	42.6 ± 1.5*	61.3 ± 1.2*	40.3 ± 1.1*	32.2 ± 1.3*
NOAEL	100	70	105	60	25	40

Notes: Data point is the mean ± SD of six replicates. The difference between means of cell viability for each H_2O_2 concentration point and control point was analysed by one-way ANOVA for each cell sample. Statistical difference between means is indicated with *asterisk* (*, $p < 0.05$). MSC sample demonstrating the hormetic model of dose response is presented in bold. The NOAEL was estimated by the dose-response curves analysis

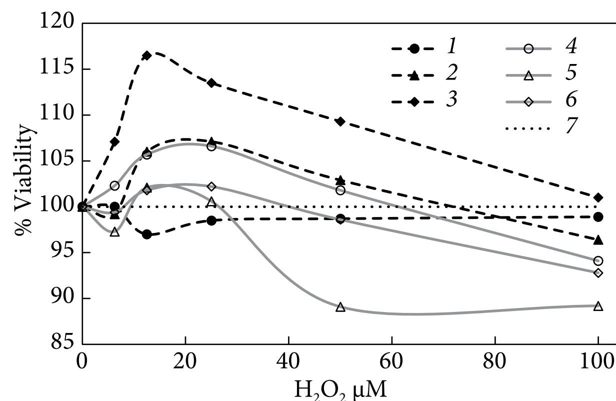

Fig. 2. The responses of WJ-MSCs to single low and moderate H_2O_2 doses ("oxidative eustress"). *1* — dWJ-MSCs (T2); *2* — dWJ-MSCs (T2); *3* — dWJ-MSCs (T1); *4* — nWJ-MSC; *5* — nWJ-MSCs; *6* — nWJ-MSCs; *7* — the dotted line corresponds to 100% cell viability

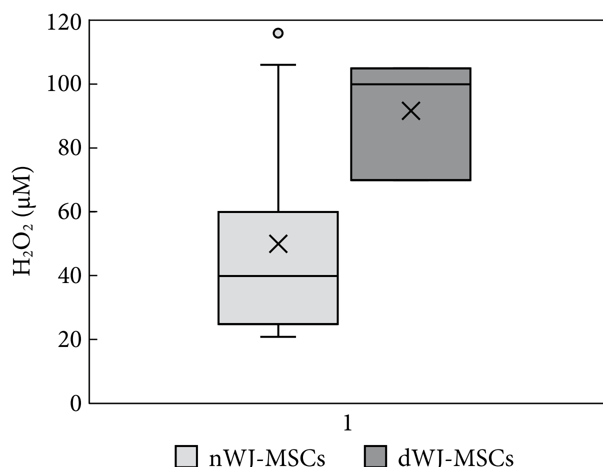

Fig. 3. Box plot of the Mann-Whitney U test for values of NOAEL Whiskers denote 1.5× the interquartile range and outliers are marked as dots.

concentrations (6,25 μM, 12,5 μM, 50 μM, 100 μM, 145 μM, 290 μM, 440 μM) for 24 h. Modelled curve of 6 WJ-MSCs pooled samples in response to different H_2O_2 doses is shown in Fig. 1. The results are expressed as survival rates, calculated as the percentage of MTT activity normalized to control (without H_2O_2). The values for the curve parameters are shown in Table 1.

The responses of WJ-MSCs in the concentration range denoted as "oxidative eustress" are detailed in Fig. 2. According to the overview of H. Sies, the intracellular physiological range of H_2O_2 spans between 1 and 10 up to approx. 100 nM (for liver cells). The approximate limits of the "oxidative eustress" range are 100—1000 nM and adaptive stress responses occur at these concentrations. Given that the cellular antioxidant pathways create a gradient between extracellular and intracellular levels of exogenous H_2O_2 of about 100-fold in the cells exposed to the micromole doses, exogenous H_2O_2 concentrations in this region range from 1 to 100 μM [17]. In this range we showed that exogenously applied H_2O_2 (6,25—100 μM, which corresponds to 60—1000 nM at the intracellular level) induced non-monotonic dose response with specific features for hormetic and threshold models. The samples 2, 3 and 4 represented dose-response curves depicting the quantitative features of hormesis, which is characterized by a low dose stimulation and high dose inhibitory effects and considered as the first quantitative estimate of biological plasticity. The samples 1, 5 and 6 represented dose-response curves depicting the quantitative features of threshold response. The threshold model is characterized by an initial zero response that extends up to a certain dose, and then damage occurs according to the dose.

We have found in the previous study [18] that there is a large inter-subject variability of WJ-MSCs in response to oxidative stress at each tested dose of H_2O_2, but only 2 types of stress responses were observed. The comparison of responses to oxidative stress induced by H_2O_2 *in vitro* between WJ-MSCs derived from diabetic and healthy mothers showed no considerable difference in mode of response and some quantitative features. For example, the maximum amplitude of the stimulation response was in the range of 6—17%, as in previous work (n = 12). However, one feature distinguished dWJ-MSCs from nWJ-MSCs. dWJ-MSCs were characterized by a higher NOAEL values (70 μM, 80 μM and 100 μM). For nWJ-MSCs the NOAEL were 25 μM, 40 μM and 60 μM. In our previous work, where cells were also studied under oxidative

stress at the same conditions, we found that NOAEL values in 9 out of 12 WJ-MSC samples were within the range of 20—50 μM [18]. Box-Plot illustrating the spread of NOAEL values in healthy (n = 15) and diabetic groups (n = 3) is presented in Fig. 3. However, no statistical differences were found in the diabetic group compared to the control group ($p > 0.05$) according to Mann-Whitney U test.

In cells chronically exposed to the diabetic microenvironment, antioxidant pathways may undergo some changes. Qenaei *et al.* [19] have shown that cells exposed to chronic oxidative influence have altered antioxidant defense mechanisms, which induce a series of adaptive responses that are distinct from those observed after acute exposure. Waheed T.O. *et al.* [20] also revealed that the levels of antioxidant enzymes of adipose tissue-derived MSCs were elevated during prolonged exposure to GOx-induced H_2O_2 compared to pulsed exposure to H_2O_2. Wanlu Su at al. found that diabetic microenvironment preconditioning of adipose tissue-derived mesenchymal stem cells enhanced their anti-diabetic, anti-long-term complications, and anti-infammatory effects in type 2 diabetic rats [21]. It is possible that further H_2O_2 exposure (acute exposure) *in vitro* of dWJ-MSCs, that have experienced prolonged oxidative stress *in vivo,* may help these cells to survive oxidative stress at higher H_2O_2 concentrations compared to the control cells, as reflected in the NOAEL values. It should be noted that such individual maternal characteristics as age, genetic variants, and the methods of drug control of glucose levels could influence MSC responses to oxidative stress. It is possible that small amount of samples in the diabetic group in our study limited the ability to detect minor differences in NOAEL values.

Conclusions

The effect of oxidative stress on the *in vitro* metabolic activity of both normal and diabetic WJ-MSCs was manifested according to the hormetic model of the response to the stressor (dose-response with stimulation by a low dose and inhibition by a high dose) and the threshold model with adaptation to low doses without stimulation. There was a trend towards higher tolerance to moderate oxidative stress in dWJ-MSCs compared with the control cells according to NOAEL values. However, no statistically significant difference in these values between groups was found, according to the Mann-Whitney U-test. Further studies on more amount of samples are needed to use this cell model for diagnostic applications and to develop criteria for donor cells.

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ВІДПОВІДІ НА ОКСИДАТИВНИЙ СТРЕС КУЛЬТИВОВАНИХ
IN VITRO МЕЗЕНХІМАЛЬНИХ СТОВБУРОВИХ КЛІТИН ВАРТОНОВОГО
СТУДНЮ, ОТРИМАНИХ ВІД МАТЕРІВ З ДІАБЕТОМ

Мета. Дані щодо впливу діабетичного мікрооточення на перинатальні стовбурові клітини досі є фрагментарними та суперечливими. Метою дослідження було оцінити вплив діабетичного мікрооточення на функціональні реакції на оксидативний стрес *in vitro* розмножених МСК Вартонового студню (МСК-ВС), отриманих від

діабетичних матерів. ***Методи.*** МСК отримували методом експлантів та культивували за допомогою стандартних методів. Оксидативний стрес індукували пероксидом водню (H_2O_2), що додавався разово. Метаболічну активність МСК-ВС визначали за допомогою МТТ-аналізу. ***Результати.*** Наші результати показали, що життєздатність МСК-ВС, що знаходились під впливом діабетичного мікрооточення та контрольних клітин, зазнавала впливу від H_2O_2 за двох-фазною (горметичною) та двох-лінійною (пороговою) моделями відповіді при концентраціях від 6,25 до 440 мкМ. МТТ-аналіз не виявляв суттєвих змін виживання після обробки H_2O_2 між клітинами, що зазнали діабетичного впливу та контрольними. Однак значення NOAEL (рівень без спостережуваного побічного ефекту) для клітин, що зазнали діабетичного впливу, становило близько 100 мкМ, що не є типовим для контрольних клітин. ***Висновки.*** Вплив оксидативного стресу на метаболічну активність *in vitro* як нормальних, так і діабетичних МСК-ВС проявлявся відповідно до горметичної моделі відповіді на стресор (дозова відповідь зі стимуляцією низькою дозою та гальмування високою дозою) та порогової моделі з адаптацією до низьких доз без стимуляції Спостерігалася тенденція до вищої толерантності до помірного оксидативного стресу в дМСК-ВС порівняно з контрольними клітинами згідно зі значеннями NOAEL. Однак, згідно з U-критерієм Манна-Вітні, статистично значущої різниці в цих значеннях між групами не виявлено. Для використання цієї клітинної моделі в діагностичних цілях та розробки критеріїв для донорських клітин необхідні подальші дослідження більшої кількості зразків.

***Ключові слова*:** МСК-ВС, цукровий діабет, оксидативний стрес.