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Donor variability of the Wharton's jelly-derived MSCs in response to oxidative stress

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Aim. Since cell survival is crucial for the therapeutic efficacy, it is important to assess the MSC inter-donor variability under oxidative conditions. This study was undertaken to examine the functional responses to the oxidative stress of Wharton jelly-derived MSCs (WJ-MSCs) from different donors. **Methods.** MSCs were obtained by the explant method and cultured according to standard methods. The oxidative stress was induced by hydrogen peroxide (H_2O_2). Using the MTT assay the treated WJ-MSCs were analyzed for metabolic activity and survival. **Results.** Our findings indicated that viability of individual WJ-MSCs was affected by H_2O_2 in a biphasic mode at concentrations between 6.25 and 440 μM for most cell samples. At lower H_2O_2 concentrations, 6.25–50 μM, an increase in the viability to 16 % was noted. The maximum stimulating effect was observed at concentrations of 12.5 μM and 25 μM depending on the donor. We found that the preconditioning of WJ-MSCs with 12.5 and 25 μM H_2O_2 for 24 h enhanced their survival under toxic H_2O_2-doses and survival rates varied for different donors. **Conclusions.** The findings indicated that the WJ-MSC responses to H_2O_2-induced oxidative stress varied for different donors. The viability of H_2O_2-preconditioned WJ-MSCs changed in a donor-dependent manner in severe environment. Consequently, to produce effective medical cells it is important to consider the donor variability in the process of oxidative MSC conditioning.

Keywords: MSCs, donor variability, oxidative stress, hormesis

Introduction

Wharton jelly-derived MSCs, despite being one of the most interesting cell populations for stem cell medicine, currently have certain limitations that reduce their therapeutic efficacy. One of the most serious problems is poor retention of transplanted MSCs at injured tissue. Transplanted MSCs migrate to the sites of inflammation and injured tissue in response

to various stimuli. These sites are characterized by high levels of inflammatory and oxidative stress mediators. This harsh environment results in a serious threat to the survival of introduced cells, especially during the first few days after transplantation. Reactive oxygen species (ROS) are constantly produced in the cells and play an important role in various cellular processes, including metabolism, homeostasis and cell signaling [1]. Excessive ROS, however, may induce oxidative stress, leading to significant damages of protein, lipid, DNA and cell death [2, 3].

A number of strategies have been proposed to improve MSC survival after transplantation. The first strategy is preconditioning, which is based on the biological concept of hormesis, where brief exposure to low doses of some toxic agents leads to the beneficial effects [4–6]. It was shown that the preconditioning of MSCs through a mild oxidative stress increases their survival and activates the signaling pathways that promote resistance of the cells to more severe stresses [7–9]. However, a number of studies *in vitro* and clinical trials have demonstrated diverse outcomes. It is shown, that there are individual differences in MSC abilities based on the inter-donor variability and the extent of variation is currently under investigation [10–13].

This study was designed to estimate an extent of variation of the functional responses to oxidative stress of human WJ-MSCs from different donors and to find out the optimal doses of H_2O_2 to increase the resistance to oxidative stress. To do this, we mimicked an oxidative microenvironment *in vitro* and exposed various WJ-MSCs to the increased concentrations of H_2O_2. The cell survival was detected by a cell metabolism assay and compared the donors according to the quantitative characteristics of hormesis. The responses of H_2O_2-preconditioned cells to harsh stress were assessed under 300μM H_2O_2. We suppose that the MSC donor variability may play a role in the effectiveness of WJ-MSC employed therapeutically, and donor-related cell variabilities require a personalized approach in expanding and pretreated processes. Without recognizing these MSC variants, the clinical outcomes can also be variable or adverse.

Materials and Methods

Isolation and culture of WJ-MSCs. The procedures of isolation and culture of WJ-MSCs were previously described. In brief, Wharton's jelly was cut into 2–3 mm pieces and seeded in a cell culture dish with DMEM-F12 containing 10 % fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37 °C, 5 % CO_2 in ambient oxygen concentration to permit the cells to migrate from the explants. After achieving about 80 % confluence, the MSCs were detached and sub-cultured to a new flask [14].

WJ-MSC phenotype determination. CD marker expression was measured by flow cytometry. The cells were stained with FITC- or PE-conjugated antibodies specific for human CD34, CD73, CD90, CD105 (Biosciences, San Jose, CA, USA) and analyzed by a FACScalibur using Cell Quest software (BD Biosciences, San Jose, CA, USA).

H_2O_2 treatment. To simulate oxidative stress, 3 % H_2O_2 was dissolved in complete medium to the following final concentrations: 6.25, 12.5, 25, 50, 100, 145, 300, and 440 μM; the control variant consisted of H_2O_2-free complete medium. To estimate the responses of the MSCs preconditioned with non-toxic concentrations of H_2O_2 (6.25, 12,.5, 25 μM) for 24 h against increased oxidative stress, all media were replaced with H_2O_2-free complete medium for 24 h. After the recovery period the culture medium was replaced with 300 μM H_2O_2-conditioned medium for 24 h. As a control, cells were treated with DMEM medium only. The cell viability was determined using the colorimetric MTT assay.

Cell metabolic assay. MTT assay which is a measure of cell survival or viability and mitochondrial metabolic activity was performed as described previously [15]. Briefly, WJ-MSCs were cultured at a density of 5000 cells/well in triplicates in the 96-well tissue culture plates. After 24 h of cell attachment, the cells were exposed to H_2O_2 in a wide range of concentration (from 6.25 μM to 440 μM) by replacing the old DMEM with fresh medium containing H_2O_2. Then the cells were incubated at normal culturing conditions for 24 h. Subsequently, the cells were treated with MTT solution (0.5 mg/mL) at 37 °C for 4 h. The formazan crystals were dissolved in 100 μL dimethylsulfoxide (DMSO). The absorbance of each well was measured at 570 nm using microplate reader.The absorbance ratios of control vs. treated cells were used for the calculation of the percent viability. The results were presented as % MTT activity where the readings for the control cells were considered as 100 % within each experiment.

Statistical analysis

OriginPro 8.5 SR1 software (OriginLab Corporation, USA) and Excel (Microsoft) were used for statistical analysis and graphic illustration. The mean values of all results were expressed as the mean ± SD. $P < 0.05$ was considered statistically significant.

Results

WJ-MSCs from 12 different donors were isolated from Wharton's jelly and expanded to P2 under identical conditions. The cultures exhibited a fibroblast-like spindle-shaped morphology and a high level of adherence to the plastic culture flasks. The cells were characterized according to International Society of Cell Therapy minimum criteria for defining MSCs. The analysis of specific cell surface markers at passage 2 revealed positive expression of CD73, CD90 and CD105 (>95 %) and negative expression of CD34 (<0.5 %). Flow cytometric immunophenotyping of the cells cultured under mild oxidative and control conditions did not show any differences in positive markers (CD73, CD90 and CD105) or negative marker CD 34.

To determine whether donor variation affects the resistance to oxidative stress of WJ-MSCs, we studied how it affected the metabolic activity of the cells in the presence of H_2O_2 in a wide range of concentrations. Once P2 was reached, H_2O_2 was used to simulate oxidative stress. The cells of 12 donors were exposed to 9 increased concentrations of H_2O_2 for 24 h (more than 70-fold range; from 6.25 μM to 440 μM). The metabolic activity of treated cells changed with increasing H_2O_2 concentration nonlineary. MTT assay showed both biphasic dose–responses of the H_2O_2-treated WJ-MSCs as one of the hormetic features and bilinear responses (threshold model). Survival of WJ-MSCs from each

donor in the presence of 6.25–100 μM H_2O_2 for 24 h either increased or did not change significantly as compared to untreated MSCs. In contrast, the exposure of WJ-MSCs to 300 and 440 μM H_2O_2 for 24 h decreased their viability up to 43.41 ± 4.16 % (#11) (Table 1) and 34.9 % ± 2.8 % (#7), respectively.

Table 1. **Individual features of dosage effects of H_2O_2 treatment on WJ-MSCs** *in vitro*

MSC sample	Maximum Stimulation (% viability)	NOAEL (μM)	Effect of 300μM H_2O_2 (% viability)
#1	115.2 ± 2.7*	21	60.2 ± 3.4
#2	111.0 ± 4.1*	54	78.1 ± 3.8
#3	**103.1** ± 4.4	35♦	77.2 ± 5.1
#4	114.8 ± 3.6*	21	47.7 ± 2.9
#5	116.3 ± 3.4*	116	61.2 ± 4.1
#6	**101.1** ± 3.3	105♦	62.4 ± 3.0
#7	**101.4** ± 2.5	25♦	57.6± 4.2
#8	106.9 ± 2.9*	45	55.9 ± 3.9
#9	113.6 ± 2.8*	106	58.0 ± 4.8
#10	**104.3** ± 3.1	43♦	56.3 ± 2.8
#11	106.2 ± 1.8*	21	43.4 ± 4.2
#12	**102.6** ± 3.1	35♦	65.1 ± 3.7

Data point is the mean ± SD of three replicates for each donor for cell viability. *designates statistical significance. P<0.05 as compared to untreated cells. ♦ designates MSC sample demonstrating the threshold model of dose response

Moreover, the donor-related variations were revealed in response to the oxidative stress. It was represented graphically by inverted U-shaped dose-response curves, which depict the quantitative features of hormesis, namely, maximum stimulation points, dose-response stimulation range, and NOAEL (no observed adverse effect level) (Fig. 1). To accentuate the extent of donor variability in MSC responsiveness to oxidative stress, the detailed dot plots were generated for the donor pool in the hormetic zone.

As to the quantitatively-based criteria, the results showed that the magnitudes of hormetic responses of the WJ-MSCs from different donors were variable and modest, ranging from 7 to 16 %, and were similar to those reported for hormetic dose responses (Table 1, Fig. 1) [4, 16]. The hormetic dose-response stimulation range was within 7- 9-fold dose range. The distances between the maximum stimulatory response and the estimated NOAEL were in the 2- to 3-fold range, except for #9 (long asymmetric hormetic zone). The NOAEL was estimated by the dose-response curves analysis.

Although MSCs can be manipulated *in vitro* via preconditioning with enhanced antioxidative effects, at present it is not known whether and how the donor-related MSC variability will affect the survival of H_2O_2-preconditioned MSCs exposed to severe oxidative stress. In order to determine whether the viability of the H_2O_2-preconditioned WJ-MSCs can be changed in a donor-dependent manner, the cells #9, #11 (hormetic response), and #10, #12 (indifferent in hormetic zone) were cultured in severe environment (300μM H_2O_2). Challenge H_2O_2 concentration of 300μM was chosen as the dose reducing control values by about 50 %, usually in a 40–60 % range according to our study and literature reports [17].

As shown in the hormesis literature, the maximum enhancement of the response is demonstrated by the dose providing the maximum protective effect [18]. The H_2O_2 doses of 12.5 μM and 25 μM were considered as optimal doses for next experiments. The results indicated that the viability of the untreated WJ-MSCs from all 4 independent samples challenged to 300 μM H_2O_2 was significantly

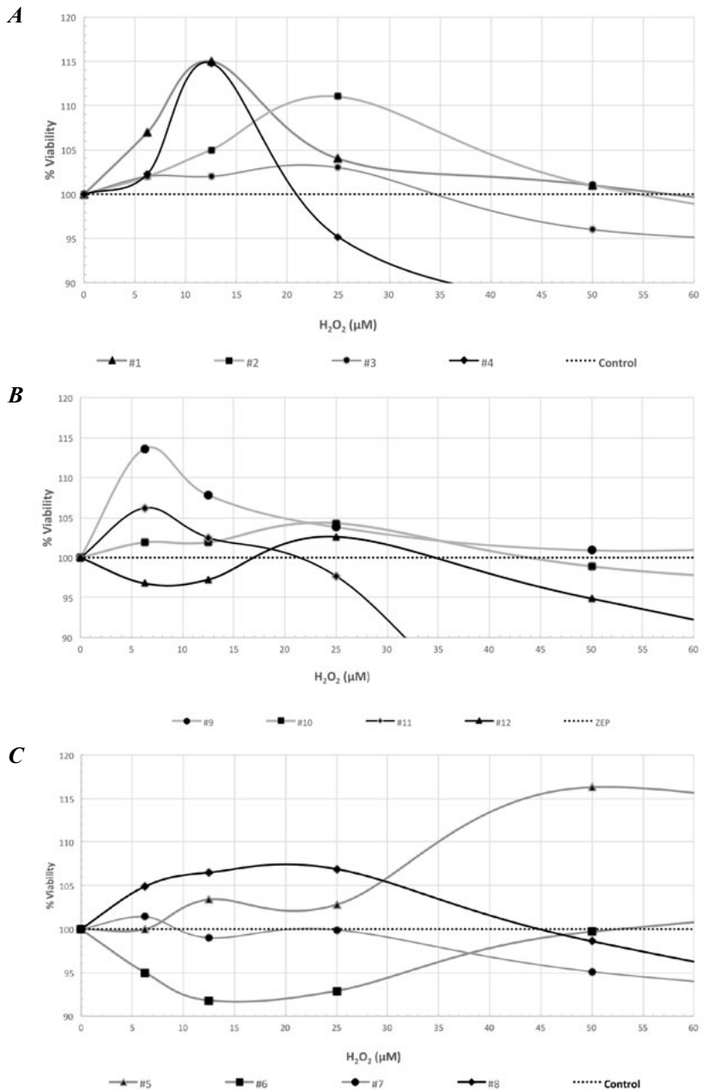

Fig. 1. Effect of H_2O_2-treatment on the cell viability of individual WJ-MSCs *in vitro* in the hormetic zone. a — #1,2,3,4. b — #5,6,7,8. c — #9,10,11,12. The results are presented as % MTT activity comparing to the untreated controls taken as 100 %.

lower compared to that preconditioned with H_2O_2. The data on pre-treated WJ-MSCs are presented in Table 2 and Figure 2. The challenging dose decreased the viability in the control cells to 58.04 %, 56.2 %, 43.4 and 65.1 %. The survival capacity of H_2O_2-preconditioned cells in adverse conditions varied, ranging from 38.6 % to 92.7 % *vs* cells

Table 2. **Response of preconditioned WJ-MSCs from different donors to severe oxidative stress**

Cell sample	12.5 μM H_2O_2 treated (% viability)	25 μM H_2O_2 treated (% viability)	Untreated cells (% viability)	Untreated cells without 300μM H_2O_2 stress
	300μM H_2O_2 stress			
#9	92.0 ± 3.89$^{\alpha}$	91.3 ± 2.97$^{\Delta}$	58.04 ± 4.85	100 ± 2.06
#10	92.7 ± 5.66$^{\alpha}$	80.21 ± 5.78$^{\Delta}$	56.2 ± 2.76	100 ± 3.24
#11	41.48 ± 3.75	40.59 ± 1.28	43.41 ± 4.16	100 ± 7.88
#12	64.46 ± 6.25	65.6 ± 1.73	65.1 ± 3.7	100 ± 1.96

Data point is the mean ± SD, n=3. $^{\alpha}$, $^{\Delta}$ $p < 0.05$ vs. untreated control for 12.5μM H_2O_2- or 25μM H_2O_2-treated WJ-MSCs, respectively.

in normal conditions. The preconditioning with a pick at 12.5 or 25 μM H_2O_2 significantly changed the cell viability to following stress in a donor-dependent manner. These doses induced cell-survival adaptive responses in WJ-MSCs #9 and #10 to the challenge dose, indicating a benefit of preconditioning. For example, 25 μM H_2O_2-conditioning treatment affected a relative increase of the protective response from 58.04 % and 56.2 % to 92 % and 92.7 %, respectively to #9 and #10. However, the cells #11 and #12 did not show any difference in viability comparing to untreated WJ-MSCs (Table 2).

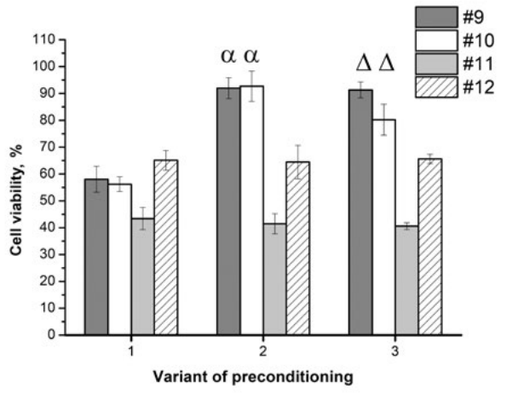

Fig. 2. The protective effect of H_2O_2 preconditioning on the viability of WJ-MSCs following oxidative stress with 300μM H_2O_2 for 24 h. **1** – non pre-incubated cells (control). Cells were pre-incubated with 12.5 μM H_2O_2 (**2**) and 25μM H_2O_2 (**3**). Data point is the mean ± SD, n=3. The vertical bar represents standard deviation of the mean. $^{\alpha}$, $^{\Delta}$ $p < 0.05$ vs. untreated control for 12.5μM H_2O_2- or 25μM H_2O_2-treated WJ-MSCs, respectively.

Discussion. The poor outcomes of stem cell transplantation have been suggested to result from residual oxidative damage affecting the transplanted cells. MSCs unable to counteract the toxic environment in which they must act have lower therapeutic potential. At present, it has been shown that MSCs exert antioxidant properties in a variety of studies *in vitro* and *in vivo* associated with high levels of ROS [**19**]. Nevertheless, the fetal MSCs are exposed to the fetal circulation and experience lower levels of inflammation and oxidative stress [20]. Therefore, for WJ-MSC-based therapy, it is critical to quantify donor variability for functionally specific MSC responses under oxidative stress. Considering that responsiveness to oxidative stress may be different for the WJ-MSCs originated from different donors, the present study focused on two purposes. The first aim was to compare the effects of

H_2O_2 as one of the most stable ROS in wide range of concentrations on the viability of WJ-MSCs derived from different donors *in vitro* in order to choose the optimal concentration for conditioning protocols. The second purpose was to assess the protective effects of preconditioning with non-toxic concentrations of H_2O_2 on the survival of individual WJ-MSCs under harsh condition in order to provide the information for the selection of cells with improved therapeutic effectiveness. H_2O_2 as a conditioning agent showed positive hormetic results in the study, so the quatitative characteristics of hormesis were determined to quantify donor variability among individual WJ-MSCs for responses to oxidative stress. However, in our study, some individual WJ-MSCs did not respond significantly to the hormetic stimulus. We decided to test these cells in severe milieu *in vitro* following H_2O_2-conditioning. To do this, the cells, which did not respond to the hormonal stimulus were challenged with toxic 300 μM H_2O_2. The result was not predictable. The sample #9, which responded to hormetic stimulus, maintained high viability under stress (92 % vs 58 % for untreated cells). Surprisingly, the conditioned cells #10 and #12 (showing no statistically significant hormetic stimulation) responded differently to harsh oxidative stress. WJ-MSCs #10 also maintained high viability under 300 μM H_2O_2 (92 % vs 56 % for untreated cells), while the cells #11 and #12 decreased their viability to the level of untreated cells. According to current literature data the pretreatment may change some MSC properties in different ways. Zhang and colleagues [21] found that pretreatment with IFN-γ and TNF-α eradicated the donor-dependent variation of the human UC-MSCs in immunomodulation and suggested that stimulation may improve the anti-inflammatory function of UC-MSCs and eliminate the donor-dependent variation. We also revealed the same effect of eradication of donor-dependent variation of WJ-MSCs #9 and #10 in response to the oxidative stress after H_2O_2 preconditioning. Nonetheless, the cells #11 and #12 did not respond to conditioning and their metabolic activity was the same as that of unconditioned cells. Interestingly, Boyt *et al.* showed that the donor itself had the largest influence on the potency of pretreated MSCs and pre-licensing with IFNγ is not always enough to overcome inherent donor weakness in immunosuppression. The authors concluded that the donor pre-selection is needed to realize the significant advantage of pre-treatment [22].

Our study also demonstrated that the process of preconditioning WJ-MSCs with H_2O_2 also requires the personal approach depending on the donor. Taken together, our findings suggest that the oxidative preconditioning cannot be applied as an enhancement strategy for some individual WJ-MSCs and inter-individual MSC variability should be taken into account.

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Донорна варіабельність мезенхімальних стовбурових клітин Вартонова студня за умов окислювального стресу

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Мета. Оскільки виживання клітин має вирішальне значення для терапевтичної ефективності, важливо оцінити донорну варіабельність МСК за окислювального стресу. Дане дослідження було проведено з метою вивчення реакцій МСК Вартонова студня людини (ВС-МСК) різних донорів на окислювальний стрес. **Методи.** МСК отримували методом експлантів і культивували за стандартними методами. Окислювальний стрес викликали перекисом водню (H_2O_2). Метаболічна активність та виживання ВС-МСК були проаналізовані за допомогою МТТ тесту. **Результати.** Наші результати показали біфазну залежність відповіді МСК від концентрації H_2O_2 для більшості зразків культур в межах 6,25–440 мкМ. За низьких концентрацій H_2O_2, 6,25–50 мкМ, відмічено збільшення життєздатності до 16 %. Максимальний стимулюючий ефект спостерігався за концентрацій 12,5, 25 та 50 мкМ залежно від донора. Ми виявили, що попереднє кондиціонування МСК за умов 12,5 та 25 мкМ H_2O_2 протягом 24 годин підвищило їх виживання за токсичних доз H_2O_2, а показники виживання варіювали між окремими донорами. **Висновки.** Результати показали донорну варіабельність відповідей ВС-МСК на окислювальний стрес, викликаний H_2O_2. Життєздатність прекондиціонованних H_2O_2 МСК за посиленного стресу змінювалась залежно від донора. Отже, важливо враховувати донорну варіабельність у процесі окисного кондиціонування МСК для отримання терапевтично ефективних клітин.

Ключові слова: МСК, донорна варіабельність, окислювальний стрес, гормезис

Донорная вариабельность мезенхимальных стволовых клеток Вартонова студня в условиях окислительного стресса

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Цель. Поскольку выживаемость клеток имеет решающее значение для терапевтической эффективности, важно оценить донорную вариабельность МСК при окислительном стрессе. Это исследование было проведено с целью изучения реакций МСК Вартонова студня человека (ВС-МСК) разных доноров на окислительный стресс. **Методы.** МСК получали методом эксплантов и культивировали стандартным методом. Окислительный стресс вызывали перекисью водорода (H_2O_2). Метаболическая активность и выживаемость ВС-МСК были проанализированы с помощью МТТ теста. **Результаты.** Наши результаты показали бифазную зависимость ответа МСК от концентрации H_2O_2 для большинства образцов культур в пределах 6,25–440 мкМ. При более низких концентрациях H_2O_2, 6,25–50 мкМ, отмечено увеличение жизнеспособности до 16 %. Максимальный стимулирующий эффект наблюдался при концентрациях 12,5, 25 и 50 мкМ в зависимости от донора. Мы обнаружили, что предварительное кондиционирование МСК при 12,5 и 25 мкМ H_2O_2 в течение 24 часов повысило их выживаемость при токсических дозах H_2O_2, а показатели выживаемости варьировали между отдельными донорами. **Выводы.** Результаты показали донорную вариабельность ответов ВС-МСК на окислительный стресс, вызванный H_2O_2. Жизнеспособность прекондиционированных H_2O_2 МСК при усиленном стрессе изменяется в зависимости от донора. Следовательно, важно учитывать донорную вариабельность в процессе окислительного кондиционирования МСК для получения терапевтически эффективных клеток.

Ключевые слова: МСК, донорная вариабельность, окислительный стресс, гормезис

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