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OPTIMIZATION OF OXIDATIVE PRECONDITIONING OF hWJ-MSCs TO OVERCOME OXIDATIVE STRESS

Aim. It has been shown that biological properties of MSCs can be modulated by preconditioning of MSCs under specific culture conditions. Our study aimed to evaluate the effects of different H_2O_2 concentrations during oxidative preconditioning of hWJ-MSCs to overcome subsequent severe oxidative stress and reduce cytotoxic effects. ***Methods***. MSCs were derived from human umbilical cord, cultured as monolayers according to standard methods. Oxidative stress was induced by hydrogen peroxide (H_2O_2). Treated WJ-MSCs were analyzed for metabolic activity and survival by MTT assay. ***Results***. Our findings indicated that preconditioning of WJ-MSCs with 10, 20, 30 and 40 μM H_2O_2 for 24 h enhanced their survival under toxic H_2O_2-doses and survival rates varied between different modes of preconditioning and levels of severe stress. The maximum protective effect was observed at 10 μM H_2O_2 preconditioning for 300 μM H_2O_2 severe stress. Simultaneouly, the maximum adaptive response to a stress level of 500 μM was detected only after preconditioning with 30 μM H_2O_2. ***Conclusions***. Our results demonstrate that the H_2O_2 preconditioning of WJ-MSCs could induce the cell-survival adaptive response to subsequent severe oxidative stress. However, benefit of preconditioning depends on the H_2O_2 concentration under preconditioning, the level of oxidative stress, and the characteristics of MSCs from a particular cell donor.

Keywords: MSCs, oxidative stress, preconditioning.

MSCs are multipotent cells with a broad spectrum of action and higher adaptability to *in vitro* cultures for expansion. Furthermore, it has been demonstrated that MSCs predominantly exert their therapeutic effects through the secretion of soluble paracrine bioactive factors and the release of extracellular vesicles and the secretome of cells may be used in clinical application instead of the cells. The secretome content of MSCs affected culture condition and can be modulated by priming the cells to enhance their therapeutic properties.

Oxidative stress (OS) is one of these conditions that is highly important in MSC therapy and regenerative medicine. Endogenously generated H_2O_2 regulates a wide range of cellular functions [1]. During oxidative stress, redox equilibrium is interrupted, damaging cellular functions [2]. High levels of reactive oxygen species (ROS) are produced during isolation, cell culture, and transplantation lead to OS, which limits the efficacy of MSCs [3]. In that case, the preconditioning of MSCs in oxidative conditions induced the secretion of various growth factors, proteins, cytokines and exosomes, which can improve the antioxidant potential of MSCs against subsequent OS [4, 5].

Wharton's jelly-derived MSCs (WJ-MSCs) represent one of the most promising MSC populations for cell- and free-cell modern therapy. Simultaneouly, it was shown that WJ-MSCs, as perinatal cells, are particularly susceptible to OS because they are in a state of rapid growth and development, related with high levels of energy production and metabolism, which produced a significant amount of ROS as a byproduct [6]. Pizzuti et al. [6] also reported that perinatal cells are exposed to OS during labor and delivery, which can lead to further increase ROS production during cell expansion *in vitro*. Rahimi B. and coworkers also noted that modulation of ROS level *in vitro* culturing process can control the MSC metabolism. ROS acts as a secondary messenger at mild physiological levels. At high levels, ROS lead to an OS condition, which results in apoptosis and cell death [3].

Exposure to hydrogen peroxide (H_2O_2) is a widely used method to cause oxidative stress and disturb redox homeostasis in cellular models [7]. Under conditions of moderate oxidative stress, cells activate adaptive mechanisms to overcome oxidative stress. Of note, the doses used for MSC conditioning are disproportionally high with regard to intracellular physiological range that probably extends between 1 and 10 up to approx. 100 nM H_2O_2 (for instance, for normally metabolizing liver cells). In addition, the gradient between extracellular and intracellular H_2O_2 concentrations is approximately 100-fold [8, 9]. For example, it has now been experimentally established that H_2O_2 concentration in human blood plasma is about 1–5 μM [10]. However, there are questions regarding the optimal H_2O_2 dosage required to induce an cell-survival adaptive response under conditions of severe oxidative stress.

Therefore, the aim of this study was to assess the effects of different H_2O_2 concentrations during oxidative preconditioning of hWJ-MSCs to overcome subsequent severe oxidative stress and reduce cytotoxic effects of ROS and compare the dose-dependent effects of preconditioning at different levels of OS.

Materials and methods

Isolation and culture of WJ-MSCs. 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 cells to migrate from the explants. After achieving about 70–80 % confluence, the MSCs were detached and sub-cultured to a new flask. All MSCs used in this study were under their second culture passage [11].

WJ-MSC phenotype determination. The CD surface markers expression was measured by flow cytometry with FACS (BD FACS Aria, USA). The FITC Mouse Anti-Human CD90, APC Mouse anti-Human CD73, PerCP-Cy™5.5 Mouse anti-Human CD105 and APC Mouse Anti-Human CD34 were used.

H_2O_2 preconditioning. 3 % H_2O_2 was dissolved in complete medium to next final concentrations: 10, 20, 30, 40, 300, and 500 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 (10, 20, 30, 40 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 and 500 μM H_2O_2-conditioned medium for 24 h. The cell viability was determined using colorimetric MTT assay.

MTT cell viability assay. Cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Briefly, WJ-MSCs ($5x10^3$ cells/well) were seeded in a 96-well plate. After 24 h of cell attachment, the cells were treated with different concentrations of H_2O_2 After treatment for 24 h, the MTT solution (0.5 mg/ml, 20 μl) was added, and cells were incubated for 4 h at 37°C. Subsequently, 100 μl DMSO was added to each well to solubilize the formazan reaction product with shaking for 10 min. The optical density (OD) was read at 570 nm on a microplate reader. The results were presented as % MTT activity where the readings for the control cells were considered as 100% within each experiment [12].

Statistical analyses. Data are expressed as mean ± SD. Statistical analyses were conducted using OriginPro 8.5 SR1 software (OriginLab Corporation, USA) and Excel (Microsoft). The level of significance was taken as $p < 0.05$ and $p < 0.01$.

Results and discussion

WJ-MSCs were characterized in the 2rd passage according to the ISCT criteria (The International Society for Cellular Therapy). The cells were plastic adherent, positive for CD105, CD90 and CD73 and negative for CD34 by FACS analysis.

According to literature data, oxidative conditioning of MSCs is carried out at a hydrogen peroxide concentration of about 20 μM. At the same time, we have shown in our previous study that there is inter-donor variability of WJ-MSCs in the sensitivity to oxidative stress and the effectiveness of H_2O_2 preconditioning [13]. Therefore, we subjected WJ-MSCs derived from one umbilical cord to 24 hours pretreatment with 10, 20, 30, and 40 μM H_2O_2. These H_2O_2 concentrations correspond to the extracellular physiological range where adaptive stress responses occur. Cell viability detected by MTT assay was 100.26±0.64 %, 97.53±3.29 %, 95.23±6.15 % and 83.44±2.6 % for 10, 20, 30 and 40 μM H_2O_2, respectively, compared to the control (100±0.89 %). Comparison of cell viability in the

presence of 10, 20, and 30 μM H_2O_2 showed no significant difference ($p>0.05$). While at 40 μM a partial decrease in cell viability was noted ($p < 0.01$). In the low concentration zone (concentration range 10–40 μM) no increase in metabolic activity was observed which indicated the absence of quantitative features of hormesis [14].

Next study was conducted to define whether the low H_2O_2 doses used for preconditioning could induce a cell-survival adaptive response in WJ-MSCs to subsequent severe OS. The protective effects of preconditioning with low dosages of H_2O_2 were checked in a concentration-dependent manner by addition of 300 μM and 500 μM H_2O_2. Data for pre-treated WJ-MSCs are presented in Table and Figure. The results demonstrated that the viability of the untreated WJ-MSCs challenged to 300 μM and 500 μM H_2O_2 was significantly decreased compared to those preconditioned with H_2O_2. The challenging doses decreased the viability in the untreated control cells to 74.58 % and 71.45 %, respectively.

We demonstrated that there are certain peculiarities of MSC conditioning with low doses of hydrogen peroxide. Survival capacity of preconditioned cells in adverse oxidative conditions varied depending on the H_2O_2 concentration used for preconditioning and level of OS. For example, 10 μM H_2O_2-conditioning treatment influenced a relative increase of the protective response from 74.58 % to 90 % at challenge dose 300 μM H_2O_2. However, at challenge dose 500 μM H_2O_2, the same preconditioning did not cause cell-survival adaptive response, while 30 μM H_2O_2-conditioning increased metabolic activity from 71.45 % to 88.1 %, indicating a benefit of preconditioning. Although MSCs from this cell donor did not respond to low-dose H_2O_2 stimulation with increased metabolic activity and decreased activity to only 74.5 % (for 300 μM H_2O_2) and 71.4 % (for 500 μM H_2O_2) compared to 50–60 % typical for most donor samples in our previous study [9], preconditioning did occur. The results showed that a more significant adaptive response of cells under severe oxidative stress could be achieved by optimizing the conditions of cell preconditioning. The optimization of metabolic priming of MSCs by the microenvironment (in particular, oxidative stress conditions) to improve their therapeutic capacity is still actual and requires individualized approaches depending on cell donor.

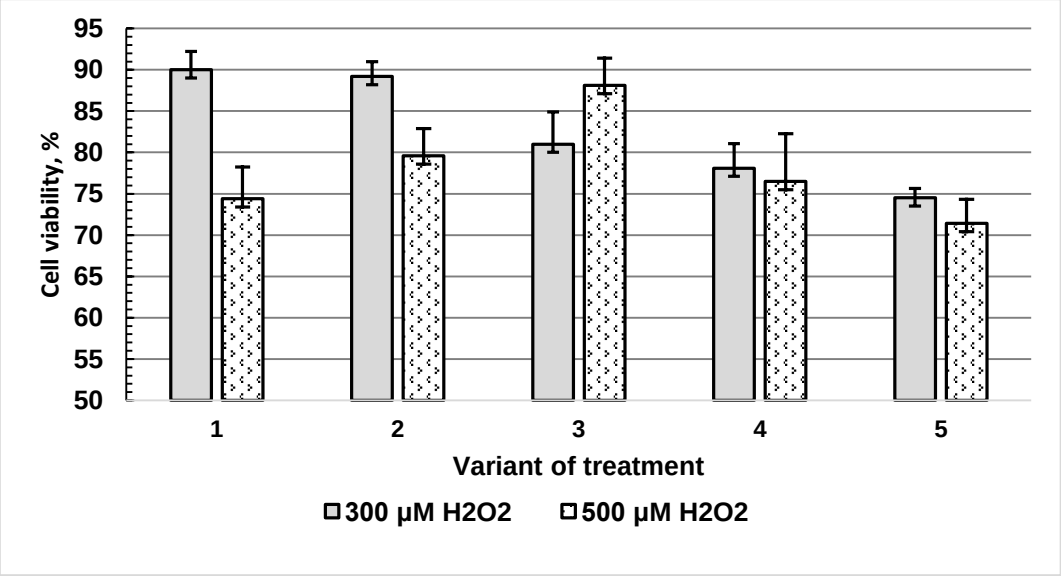

Fig. The protective effect of H_2O_2 preconditioning on the viability of WJ-MSCs following oxidative stress with 300μM and 500 μM H_2O_2 for 24 h. Cells were pre-incubated with 10 μM H_2O_2 (**1**), 20μM H_2O_2 (**2**), 30 μM H_2O_2 (**3**), and 40 μM H_2O_2 (**4**). **5** – Untreated cells +OS. Data point is the mean ± SD, n = 3. The vertical bar represents standard deviation of the mean.

Table. Response of preconditioned WJ-MSCs to severe oxidative stress

Level OS	Preconditioned WJ-MSCs (% viability)				Untreated cells +OS	Untreated cells without OS
	10 μM H_2O_2	20 μM H_2O_2	30 μM H_2O_2	40 μM H_2O_2		
300 μM	90 ± 2.23**	89.2 ± 1.8**	81.04 ± 3.89*	78.01 ± 2.97	74.58 ± 1.16	100 ± 2.01
500 μM	74.47± 3.83	79.58± 3.3*	88.1 ± 3.33**	76.46 ± 5.78	71.45 ± 2.93	100 ± 2.01

Notes: Data point is the mean ± SD, n = 3, *p < 0.05 and **p < 0.01 vs. untreated control (untreated cells + OS) for 300 μM H_2O_2 and 500 μM H_2O_2 OS (untreated cells + OS), respectively.

Conclusions

Our results demonstrate that the H_2O_2 preconditioning of WJ-MSCs could induce the cell-survival adaptive response to subsequent severe oxidative stress. However, benefit of preconditioning depends on the H_2O_2 concentration under preconditioning, the level of oxidative stress, and the characteristics of MSCs from a particular cell donor.

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ОПТИМІЗАЦІЯ ПРЕКОНДИЦІЮВАННЯ МСК ВАРТОНОВОГО СТУДНЮ ДЛЯ ЗАПОБІГАННЯ ОКИСНОГО СТРЕСУ

Мета. Показано, що біологічні властивості МСК (мезенхімальних стовбурових клітин) можна модулювати шляхом прекондиціонування за спеціальних умов культивування. Метою нашого дослідження була оцінка впливу різних концентрацій H_2O_2 для прекондиціонування МСК Вартонового студню (МСК-ВС) для захисту від подальшого посиленого окисного стресу та зменшення цитотоксичних ефектів. ***Методи***. МСК були отримані з пуповини людини та культивовані за стандартними методами. Окисний стрес був викликаний перекисом водню (H_2O_2). Метаболічну активність та виживання кондиціонованих МСК-ВС оцінювали за допомогою МТТ аналізу. ***Результати***. Наші результати показали, що попереднє кондиціонування МСК-ВС з 10, 20, 30 і 40 мкМ H_2O_2 протягом 24 годин підвищило їх виживання під токсичними дозами H_2O_2, а рівень виживаності варіювався в залежності від різних режимів попереднього кондиціонування та рівнів посиленого стресу. Максимальний захисний ефект спостерігався при попередньому кондиціонуванні 10 мкМ H_2O_2 для стресу 300 мкМ H_2O_2. Водночас максимальна адаптивна реакція на рівень стресу 500 мкМ була виявлена лише після попереднього кондиціонування 30 мкМ H_2O_2. ***Висновки***. Наші результати демонструють, що H_2O_2 попереднє кондиціонування МСК-ВС може індукувати адаптивну реакцію виживання клітин до наступного посиленого окисного стресу. Однак переваги попереднього кондиціонування залежать від концентрації H_2O_2 під час попереднього кондиціонування, рівня окислювального стресу та характеристик МСК конкретного донора клітин.
Ключові слова: МСК, окисний стрес, прекондиціонування.