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Comparison of proliferative activity of Wharton jelly mesenchymal stem cells in cultures under various gas conditions

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Aim. To optimize the cultivation of Wharton jelly-derived mesenchymal stem cells (WJ-MSCs) using physiological oxygen concentrations, and to compare the effect of "hypoxic" gas mixtures, based on nitrogen and argon, on their proliferative activity. **Methods**. From the first passage, WJ-MSCs were cultivated during five passages in the nitrogen-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % nitrogen) and argon-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % argon), 7 days before replating. At each passage the final cell number was estimated and the number of population doublings was calculated. **Results**. The proliferation level of WJ-MSCs, cultured in both gas mixtures with 3 % of O_2, was significantly higher compared to that under the regular CO_2-incubator conditions. In argon-based mixture, the WJ-MSCs proliferation was higher than in the control but lower than in nitrogen-based mixture. **Conclusion**. Cultivation of human WJ-MSCs under 3 % O_2 had a stimulating effect on the cell proliferation potential. The highest intensity of the cell multiplication was observed in the nitrogen-based mixtures.

Keywords: mesenchymal stem cells, Wharton jelly, hypoxia, physiological oxygen concentration, proliferation.

Introduction

Numerous experimental works and theoretical analysis, focused on mesenchymal stem cells (MSCs), allow considering them not only as an interesting object of studies on fundamental processes of fetal and adult life, but also as a key element in various methods of regenerative therapy. A promising position of MSCs in the cell-based therapeutic strategies results from their high proliferative and differential potential, unique paracrine effects and immune properties [1, 2, 3].

MSCs were first identified in bone marrow and described as a population of non- hematopoietic multipotent cells [4, 5]. Further studies showed that the cells with similar properties can be found in both tissues of adult organism and birth-associated tissues: amnion, placenta and umbilical cord [6]. According to the current literature, the latter are often defined as the «perinatal» stem cells, possessing the properties of both adult and embryonic stem cells [7]. Among them, MSCs from umbilical cord matrix – Wharton jelly (WJ-MSCs), are thought to be especially attractive. The formation of WJ-MSCs population at the early stages of embryogenesis [8], permits them to preserve the features of the embryonic stem cells [7, 9, 10], prominent differentiation [11], immune [12] and paracrine properties [13]. At the same time, they possess the characteristics of adult somatic mesenchymal multipotent stromal cells [14], determined by the International Society for Cellular Therapy [15].

One of the most important characteristics of MSCs, particularly for the clinical usage, is their prolifera-

tive activity. For instance, according to the literature, during the treatment of acute «graft-versus-host» disease, patients received 2×10^6–8×10^6 MSCs/kg body weight [16]. The percent of MSCs in their classical source – bone marrow for the newborns is 0.01 %, and decreases to 0.001–0.005 % with aging [17]. Therefore, it is vitally important to develop the cultivation technologies, which would allow maximal cell multiplication with preservation of the MSCs therapeutically relevant properties. The complexity of this task is related to the process of cultivation itself. Some works report that a long-term cultivation increases the risks of genetic abnormalities [18], thus a reasonable approach to provide the most effective MSCs cultivation should grant obtaining the maximal number of cells while minimizing the duration of culturing.

In the organism, a crucial role in the regulation of MSCs behavior and preservation of their properties belongs to their natural site of localization – «stem cell niche». Its components include the extracellular matrix, surrounding cells and signal molecules, produced by them. One of the key factors of niche regulation is the oxygen concentration, which is generally lower comparing not only to ambient atmospheric concentration , but also to that in other regions of the tissue. For instance, the oxygen concentration in bone marrow ranges from 2 % to 7 %, depending on the distance from capillary, and MSCs locate in the areas remote from vessels , which are, the most «hypoxic» zones [19]. Thus, the generally accepted environmental conditions of CO_2-incubator, where the oxygen concentration is similar to atmospheric, are actually «hyperoxic» for MSCs, which inevitably leads to the oxidative damages [20, 21]. Taking this into account, the MSCs cultivation at physiological oxygen concentrations, often referred to as «hypoxic», is considered to be a perspective approach [22–24].

The studies focused on the influence of hypoxic conditions on the MSCs cultivation reported their beneficial effect on multiplication, reducing oxidative stress, and engraftment in the transplantation [25–28]. However, it is hard to compare the results obtained in different works. MSCs, used in various studies, originate from different sources, the design of experiments varies from short-term preconditioning to a long-term cultivation, and the O_2 concentrations used range from 1.5 % to 8 %. The works on the influence of hypoxic conditions on WJ-MSCs, still remain rare cases.

Generally, the gas mixtures used for cultivation include nitrogen as a major «filling» component [29–31]. However, recent works have shown the cytoprotective effect of noble (or inert) gases (argon and xenon) on the cell cultures [32]. Taking this into account, we hypothesized, that using the «hypoxic» gas mixture based on the noble gas would enhance the beneficial effect of physiological oxygen concentration on the MSCs culture.

Thus, the aim of the present work was to optimize the MSCs cultivation using physiological oxygen concentrations, and to compare the effects of «hypoxic» nitrogen- and argon-based gas mixtures on the human WJ-MSCs proliferation.

Materials and Methods

MSCs were obtained from WJ of umbilical cord (UC) from three healthy donors (39–40 weeks of gestation, normal delivery), after obtaining the informed written consent, in Kyiv maternity clinic N 5. The cells were isolated using the explant method [33]. The UC fragment (5–10 cm) was washed with PBS, the vessels were mechanically removed. WJ was mechanically sliced, the fragments were placed in the cultural flacks, 75 cm^2, containing complete growth medium (DMEM with low glucose (PAA Austria) supplemented with 10 % fetal bovine serum (PAA, Austria), glutamine 2 mM (PAA, Austria), penicillin 100 U/ml (Arterium, Ukraine), streptomycin 100 μg/ml (Arterium, Ukraine). The first adherent cells were visible on 7–10 day. After 14 days the clones reached 70–80 % confluence, and the cells were passed using trypsin-EDTA (0.1 % trypsin and 0.02 % EDTA) solution. At the first passage the cells were characterized for the surface marker proteins CD90, CD73, CD105 expression (over 85 % positive), using flow cytometry (BD FACS Aria) with fluorescein- and rhodamine-conjugated antibodies (UsBiological, USA). For microscopy, inverted microscope Leica DMIL was used.

From the first passage, MSCs were seeded on plastic flacks (25 cm^2) at a density of 75,000 per flack and cultivated during 5 passages in the nitrogen-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % nitrogen) and argon-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % argon), 7 days before replating. The control group was maintained under standard CO_2 incubator conditions. For creating the hypoxic conditions, the cultural flacks with cells were placed in polyethylene bags with hermetical clasp «ZipLock». The bags were washed twice with the oxygen-free gas mixture, containing 4 % CO_2 and 96 % nitrogen or argon (depending on the group), and after that were filled with the cultivation gas mixture (see above). The bags were placed in the vacuum containers (Scarlet). The percentage «liquid media/gas» for normal gas exchange must be 1:100 [34], the volume of gas mixture must be no less than 0.7 l. The volume of bags used was 1.5 l.

At each passage, after 7 days of culturing, the cells were replated using trypsin-EDTA (0.1 % trypsin and 0.02 % EDTA) solution and counted in hemocytometer (Goryaev chamber). Population doubling was calculated as: PD = log (Nf/Ni)/log 2, where Nf – final cell number; Ni – initial cell number [22].

The data on cell numbers are represented as mean ± standard deviation for 3 samples. Statistical significance was determined using Mann-Whitney U-test at $P < 0.05$.

Results and Discussion

WJ-MSCs were expanded for five consecutive passages under 3 % O_2, in the gas mixtures based on nitrogen, argon, and ambient O_2 concentration (nearly 20 %) in CO_2-incubator. In order to assess the proliferation activity, the cells were counted at each passage. The results, summarized in Table 1 an Figure 1, show a final number of cells after 7 days of cultivation.

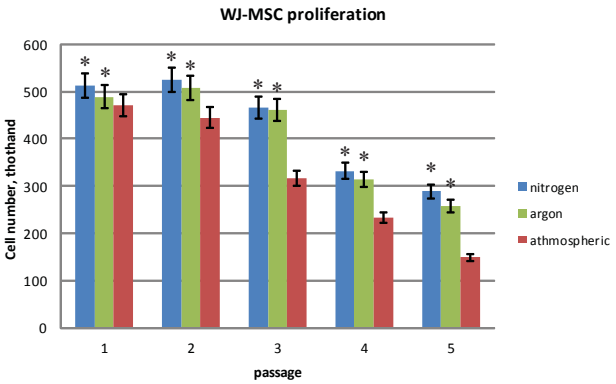

Fig. 1. Numbers of cells in WJ-MSCs cultures at 1–5 passages, after 7 days of cultivation. «Nitrogen» – nitrogen-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % nitrogen), «argon» – argon-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % argon), «atmospheric» – CO_2 incubator conditions. * – $P < 0.05$)

The proliferation level of WJ-MSCs, cultured at 3 % O_2, in both gas mixtures was significantly higher compared to that of WJ-MSCs under the CO_2-incubator conditions. This was observed at each passage.

Interestingly, a comparison of the culture growth rates at different passages within each group allows distinguishing two separate «phases» during the cultivation. The first «phase» – at the first and second passages the cultures had practically similar proliferation levels. The second «phase» – beginning from the third passage, a progressive decrease in proliferation can be observed with each new passing. For WJ-

Table 1. **Numbers of cells in WJ-MSCs cultures at 1–5 passage, after 7 days of cultivation. «Nitrogen» – nitrogen-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % nitrogen), «argon» – argon-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % argon), «atmospheric» – CO_2 incubator conditions ($P < 0.05$)**

N of passage	Nitrogen	Argon	Atmospheric
1	513.68 ± 9.58	488.3 ± 20.91	471.72 ± 9.7
2	525.47 ± 7.32	508.6 ± 25.69	444.58 ± 19.07
3	465.6 ± 2.91	460.67 ± 21.06	317.53 ± 25.23
4	332.39 ± 15.16	315.27 ± 16.22	233.62 ± 5.1
5	288.93 ± 11.39	258 ± 26.17	149.77 ± 36.93

MSCs, cultured in the gas mixtures, this tendency was less pronounced.

At the first passage of culture under 3 % O_2 in the nitrogen-based and argon-based gas mixtures, the final number of expanded WJ-MSCs was respectively 6.8 and 6.4 times higher, was higher comparing to cells from control group (6.1-fold increase). The results for the second passage were 6.9, 6.4 and 6 respectively. The data for the entire culture duration are summarized in Table 2.

The PD estimation showed that, despite a general decrease in the number of PD up to the 5th passage, the MSCs cultured under the physiological oxygen tension, had generally higher intensity of division (Table 3).

The proliferative activity is an important criterion for an estimation of the state of culture, and a sufficient activity is necessary for practical application. It strongly depends on the conditions of cultivation, each factor of environment having its own mechanisms of influence. In this context, proliferation itself can be considered a subject of research. In the present work, the proliferation rates of MSCs cultures, expanded under physiological oxygen tensions – 3 %, in the mixtures based on nitrogen and argon, were validated. The estimation of cells number at 1–5 passages showed significantly higher numbers in the gas mixtures at each passage, comparing to the groups from control CO_2-incubator. The results also revealed a higher level of PD under hypoxic conditions up to the 5th passage. The effect of mild hypoxia appeared to be stimulating in both mixtures, at the same time, the differences between these conditions were detected.

Table 2. **Multiplication in WJ- MSCs cultures at 1–5 passages, n-fold increase in number after 7 days of cultivation**

Passage	Nitrogen	Argon	Atmospheric
1	6.8	6.4	6.1
2	6.9	6.8	6
3	6	6	4.1
4	4.4	4.2	3.1
5	3.8	3.4	1.9

N o t e. «Nitrogen» – nitrogen-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % nitrogen), «argon» – argon-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % argon), « atmospheric» – CO_2 incubator conditions ($P < 0.05$)

Table 3. **Population doublings in WJ-MSCs cultures at 1–5 passages, after 7 days of cultivation**

N of passage	Nitrogen	Argon	Atmosperic
1	2.76	2.68	2.61
2	2.79	2.76	2.59
3	2.59	2.59	2.04
4	2.15	2.07	1.64
5	1.95	1.78	1

N o t e. «Nitrogen» – nitrogen-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % nitrogen), «argon» – argon-based gas mixture (3 % oxygen, 4 % carbon dioxide, 93 % argon), « atmospheric» – CO_2 incubator conditions ($P < 0.05$)

Although a high variability in the object and methods of studies on hypoxia complicates the comparison of results, we can assume that the tendencies, observed in the present work, are generally in line with those described in literature. Nevertheless, it is important to point out the differences. The work by Basciano et al. revealed the slowing of culture growth at early passages under hypoxic conditions [22]. We have not observed any similar effect. Ren et al detected the morphological signs of accelerated cellular senescence in hypoxic conditions, probably associated with a higher population doublings number [35]. In the present study there was no evidence of degenerative changes of morphology in the cultures expanded under hypoxic conditions (data not shown).

The current literature distinguishes several potential mechanisms of physiological oxygen concentrations impact. First, the reactive oxygen species are one of the major sources of DNA damage [36]. Hypoxic conditions were shown to prevent the accumulation of damages of genetic apparatus, which in control groups can be detected since the second passage [17]. This can play an important role during long-term cultivation. Next, the researches show that hypoxic conditions increase the level of cytokine receptors expression, as well as the production of growth factors. In this case, hypoxia « sensibilizes « the cells to serum growth factor, and to that produced by the cells themselves [27]. Next, there are data

showing that hypoxia activates the signal cascades of cell survival. The cultures in hypoxic conditions are shown to have lower levels of necrosis comparing to those cultured under the ambient oxygen concentration [24]. Each of these mechanisms can contribute to the general effect of physiological oxygen concentration, and determination of their roles is still considered the question of interest.

To date, the data about the effect of noble gases on MSCs culture are still lacking. There are only few works conducted to determine the influence of inert gases (xenon and argon), which demonstrated a cytoprotective effect on the cell cultures of neural origin. For example, the modeling of ischemic and traumatic damages of hippocampal slice culture in gas mixtures, containing various concentrations of argon (25 %, 50 % and 74 %) and atmospheric oxygen concentration (21 %), showed a decreased level of cellular death compared to the control samples. The underlying mechanisms are unknown [32]. Though, Fahlenkamp *et al.* demonstrated the activation of ERK 1/2, kinase that plays an important role in the processes of cells proliferation and survival, in the primary cultures of mouse embryonic astrocytes and neurons, cultured under 20 % O_2 and 50 % argon [37]. It is possible that MSCs possess the similar mechanism.

The present work showed that the level of proliferation of WJ-MSCs, cultured in argon-based gas mixtures was higher comparing to WJ-MSCs from CO_2-incubator conditions, but lower than that for the cultures from nitrogen-based mixtures. To explain these results, further research is required. Besides, taking into account the variety of existing cultivation protocols, changing the cultural strategy could probably lead to a different effect.

Conclusions

Cultivation of human WJ-MSCs under 3 % O_2 in gas mixtures, based on nitrogen and argon, had a beneficial effect on the cells proliferative activity and preservation of multiplication potential. The hypoxic argon- and nitrogen-based gas mixtures had different effects. The highest intensity of cell proliferation was observed in the nitrogen-based mixtures.

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Порівняльна оцінка ростових показників мезенхімальних стовбурових клітин Вартонового студня в різних газових сумішах

Н. С. Шувалова, В. А. Кордюм

Мета. Оптимізувати культивування і порівняти вплив газових сумішей зі зниженим вмістом кисню, на основі азоту та аргону, на процеси проліферації в культурах мезенхімальних стовбурових клітин Вартонового студню (МСК-ВС). **Методи**. Протягом 5 пасажів. МСК-ВС культивували в газових сумішах на основі азоту (кисень – 3 %, вуглекислий газ – 4 %, азот – 93 %) та аргону (кисень – 3 %, вуглекислий газ –

4 %, аргон – 93 %). На кожному пасажі після 7 днів культивування підраховували кількість клітин, і визначали число подвоєнь культури. **Результати**. Чисельність клітин МСК-ВС, культивованих в сумішах, що містять 3 % кисню, була вищою, ніж в загальноприйнятих умовах CO_2-інкубатора. Рівень проліферації в суміші на основі аргону був нижчим, ніж в суміші на основі азоту, при цьому будучи вищим за такий для контрольних груп. **Висновки**. Культивування в газових сумішах, що містять 3 % кисню, мало стимулюючий вплив на процеси проліферації МСК-ВС. Найбільш високий рівень мультиплікації спостерігали в сумішах на основі азоту.

Ключові слова: мезенхімальні стовбурові клітини, Вартонів студень, гіпоксія, фізіологічні концентрації кисню, проліферація

Сравнительная оценка ростовых показателей мезенхимальных стволовых клеток Вартонова студня в различных газовых смесях

Н. С. Шувалова, В. А. Кордюм

Цель. Оптимизация культивирования и сравнение влияния газовых смесей с пониженным содержанием кислорода на основе азота и аргона на процессы пролиферации в культурах мезенхимальных стволовых клеток Вартонова студня (МСК-ВС). **Методы**. На протяжении 5 пассажей. МСК-ВС культивировали в газовых смесях на основе азота (кислород – 3 %, углекислый газ – 4 %, азот – 93 %) и аргона (кислород – 3 %, углекислый газ – 4 %, аргон – 93 %). На каждом пассаже после 7 дней культивирования подсчитывали количество клеток, и определяли число удвоений культуры. **Результаты**. Численность клеток МСК-ВС, культивированных в смесях, содержащих 3 % кислорода, была выше, чем в общепринятых условиях CO_2-инкубатора. Уровень пролиферации в смеси на основе аргона был несколько ниже, чем в смеси на основе азота, при этом будучи выше такого показателя для контрольных групп. **Выводы**. Культивирование в газовых смесях, содержащих 3 % кислорода, оказывало стимулирующее влияние на процессы пролиферации МСК-ВС. Наиболее высокий уровень мультипликации наблюдали в смесях на основе азота.

Ключевые слова: мезенхимальные стволовые клетки, Вартонов студень, гипоксия, физиологические концентрации кислорода, пролиферация.

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