The influence of hydroplasma on the proliferative potential of human mesenchymal stromal cells (hMSCs) culture

Eremin PS[1,*], Gilmutdinova IR[1], Kostromina EYu[1], Vorobyova IG[1], Yakovlev MYu[1], Gilmutdinova FG[2], Alekseeva SV[3], Kanovsky EI[4] and Inyushin VM[3].

[1]*National Medical Research Centre for Rehabilitation and Balneology, Russian Ministry of Health, 121099, Moscow, Novy Arbat, 32*
[2] *Family Health Center "MiR", 460040, Orenburg, Mira street, 62*
[3] *Al-Farabi Kazakh National University, 950040/A15E3B4 Kazakhstan, Almaty city, Bostandyk district, Al-Farabi Avenue, 71/19*
[4] *50031, TOO "ORGANIC PRODUCTION" Kazakhstan, Almaty city, Str. Tole bi, 304.*
** Corresponding author. Email: ereminps@gmail.com*

*ereminps@gmail.com

Abstract. The present paper reviews the influence of the biologically active supplement “Hydroplasma” on the proliferative potential of hMSCs culture. The following parameters were assessed: cytotoxicity of the studied drug, cell population doubling time, study of cellular migration and cell cooperation (Wound Healing Assay), and secretory activity of cell culture. Statistical data processing was performed using IBM SPSS Statistics 19 package.
It was proved that in hydroplasma “Water for life” (in concentrations recommended by the manufacturer) and in hydroplasma “Absolute Energy” (in extremely low concentration) there was no statistically significant difference in proliferative and secretory activity of cell culture in comparison with the observational culture of hMSCs. When estimating the total impact of AE hydroplasma in standard concentrations, the death of individual hMSCs was observed from the first day of cultivation. By the fifth day after the beginning of the experiment, all the cells were dead. It was associated with a low content of TNFα, IL-6/-8 in the conditioned environment, as well as a minor increase in the amount of IL-10.

1. Introduction

Damage of cell membranes and other cell structures by free oxygen radicals is one of the reasons of pathological processes in the organism that lead to various somatic diseases. Furthermore, as the organism ages, the activity of free radicals increases, resulting in oxidative stress of cell structures (1-2).

Oxidative stress is a chemical intracellular condition that reflects an imbalance between ROI formation (free radicals and peroxides) and the capability of cells to clear them through the antioxidant system (AOS) (glutathione, ascorbic acid, tocopherol, carnitine, pentoxifylline, etc.) (3). In order to correct the processes of free-radical oxidation (FRO) in various diseases drugs with antioxidant activity are used. All antioxidants (AO) are classified as indirect and direct acting drugs. Indirect-acting AO exhibit activity in vivo and are ineffective in vitro. They stimulate AOS and can reduce FRO intensity. Direct-acting AO have pronounced antiradical properties, which are determined during in vitro testing. The majority of drugs with antioxidant effect belong to this group (4-6).

Lately, there has been a significant increase in interest in herbal, natural drugs (nutraceuticals, parapharmaceuticals) as they are safer and more adjusted to human physiology. One of the most common natural compounds is bioflavonoids, the largest class of plant polyphenols (7). Among the numerous biological supplements with bioflavonoids we can single out the so-called “Hydroplasma”. According to the manufacturer, "Hydroplasma" is a concentrate for the preparation of biogenic water, which increases the degree of biological activity of any fluid, streamlining its structure, creating the effect of structuring bioplasm with energy-intensive biogenic memory of “living water”. This product neutralises the impact of radicals, restores the central and nervous system, muscular system; it improves brain function by 20–25%, increases strength under physical activity, increases endurance and work capacity, fills the body with energy, improves concentration and memory. It neutralises all sorts of dependencies and is essential at high mental buoyancy, physical trainings, mountain climbing and in areas where human efforts are needed. This concentrate is required for treating a wide range of diseases, for recovery from severe conditions. It promotes early recovery, exhibits preventive properties, provides health, youth, energy, stress resistance and longevity.

All aforesaid served as a reason to study the efficiency and safety of in vitro hydroplasma on hMSCs cell culture.

Materials and methods.

The influence of hydroplasma on the proliferative potential of human mesenchymal stromal cells (hMSCs) culture has been studied using hMSCs market cell line (Sigma-Aldrich (Merck), Lot.: 492-05A, 2nd passage). Cell mass growth was conducted according to the manufacturer's recommendations using the growth medium intended for hMSCs cultivation. Study objects, Absolute Energy (AE) Hydroplasma and Water for Life (WoL) Hydroplasma were diluted in a growth medium according to the instructions to the concentration of ×1, ×0.5 and ×2. For AE Hydroplasma, maximum dilution of $\times 10^{-5}$ was included in the study.

To estimate the proliferative potential, cell culture was subcultured onto 6-basin titer plates at the rate of 10^4 c/cm^2. Further, the studied drugs were added in different concentrations and cultivated during 5 days. Cell growth was made visible every 24 hours via Axiovision software using Zeiss Axio Observer A1 inverted microscope with AxioCam MRC5 digital camera. Upon completion of cultivation, cells were removed from the plastic surface with trypsin solution with 0.25% EDTA (StemCellTechnology, USA). The calculation was performed with viability assessment using Bio-Rad TC-20 automated cell counter (Bio-Rad, USA) according to the manufacturer's methodology and the time of cell population doubling (DT) was calculated by the formula: $TD = (\log_2 2)\times t/[\log_2(N/N0)]$, where t is population growth time, N is the number of cells over time t, N0 is the initial the number of cells.

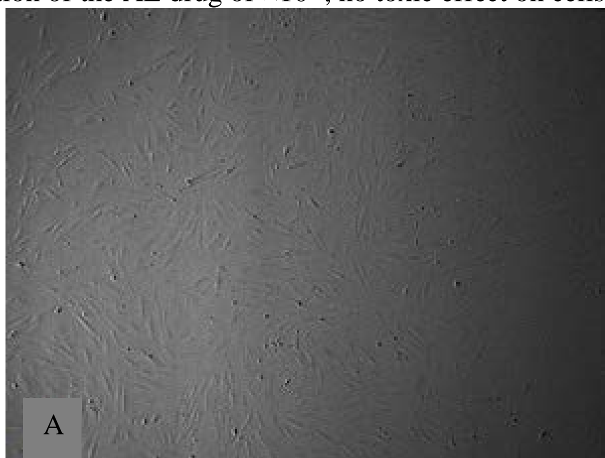
The Wound Healing Assay method was used to study cellular migration and cell cooperation. Cell culture was subcultured onto 6-basin titer plates at the rate of 10^4 c/cm^2. Further, it was cultivated until single layer formation was formed. After that, a mark was made using a sterile serological pipette tip. Then the studied drugs were added to the culture medium. The changes were then visualised with a microscope.

To assess the secretory activity at the end of the proliferative potential experiment, hMSCs cultivation medium was examined. The resultant conditioned media was carefully sampled in separate labelled vials and frozen at -20°C. After obtaining samples of the conditioned media, the secretory activity of cells in the culture medium was evaluated using the Vector-Best ELISA kits (Russia) according to the manufacturer's method.

Statistical data processing was performed using IBM SPSS Statistics 19 package. A pair-wise comparison of groups was analysed using Mann-Whitney non-parametric criterion. The data presented in the findings and discussion are presented as Me [Q1; Q3].

Findings and discussion.

In visual assessment, the toxicity of the WoL drug was not noted. Cells grow well in the presence of the drug. During coculturing with WoL, the observational group demonstrates some advance in growth rate on day 3. There was no visual difference between the dilutions ×0.5, ×1, ×2. In this regard, it was decided to continue further experiment with ×1 dilution. When assessing the cell culture growth with AE drug in ×0.5, ×1, ×2 dilutions, the hMSCs cell culture death was observed. This was probably due to the presence of photodynamic active components having blue-greenish tint in the drug composition. When the maximum dilution of the AE drug of $\times 10^{-5}$, no toxic effect on cells was observed (Fig. 1).



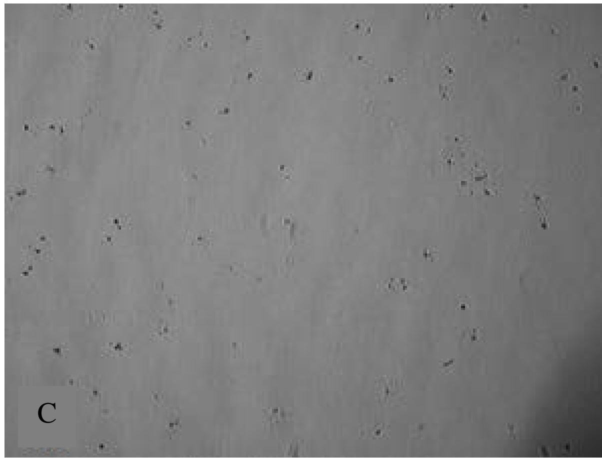
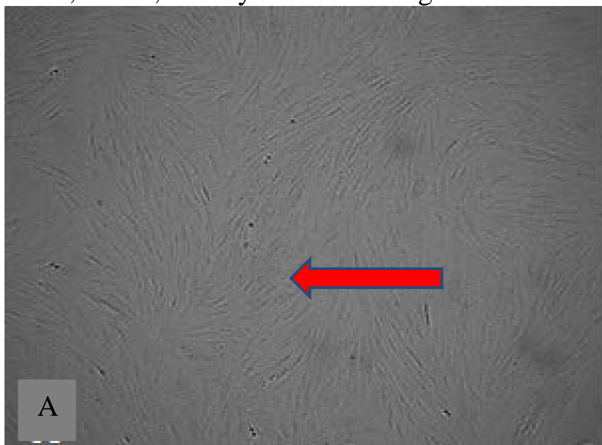
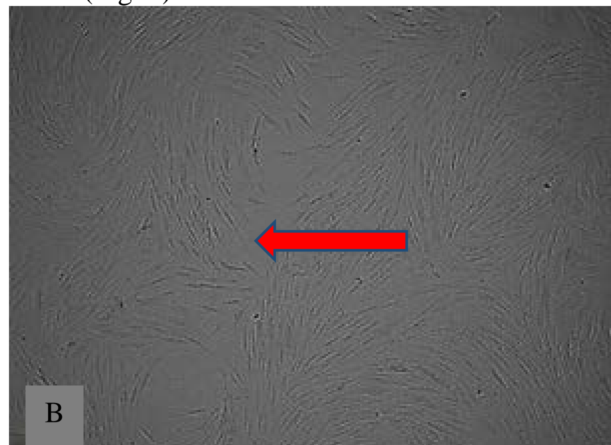


Fig. 1. Picture of hMSCs cell culture in the medium on the 5th day of cultivation. A—native culture of hMSCs cells. B—hMSCs cell culture in a medium containing the studied WoL drug in concentration ×1. C—hMSCs cell culture in a medium containing the studied drug AE in concentration ×1. D—hMSCs cell culture in a medium containing the studied AE drug in concentration $\times 10^{-5}$. Inc. × 50 l

During the study of proliferative activity, the dynamics of growth of experimental and observational hMSCs cell cultures were found to be similar. The number of cells obtained from one cultural glassware in the observational group was 221[197;237.5] $\times 10^3$, compared to 210[193.5;223.5] $\times 10^3$ cells in the experimental WoL group at a dilution rate of x1 and 212.5[190.75;242] $\times 10^3$ in the AE group at concentration of 10^{-5}. The time of cell population doubling (DT) for the hMSCs observational group was 54.77[52.62;64.23] hours, which corresponds to the literature data. When cultuvating cells in media containing the WoL drug, DT was 56.57[54.67;63.61] hours. Due to the fact that no viable cells were found in cell culture of hMSCs, cultivated in the medium containing AE drug in standard concentrations, the cell population doubling time was not calculated. With AE concentration of $\times 10^{-5}$, the proliferation rate of hMSCs cells was 55.9[52.23;65.33] hours. There was no statistically significant difference between the observational cell culture and cells cultured in media containing WoL, which presupposes the same rate of cell culture proliferation.

To visually demonstrate the proliferative activity of hMSCs cell culture, the Wound Healing Assay was conducted, that is, a study of cellular migration and cell cooperation (Fig. 2).





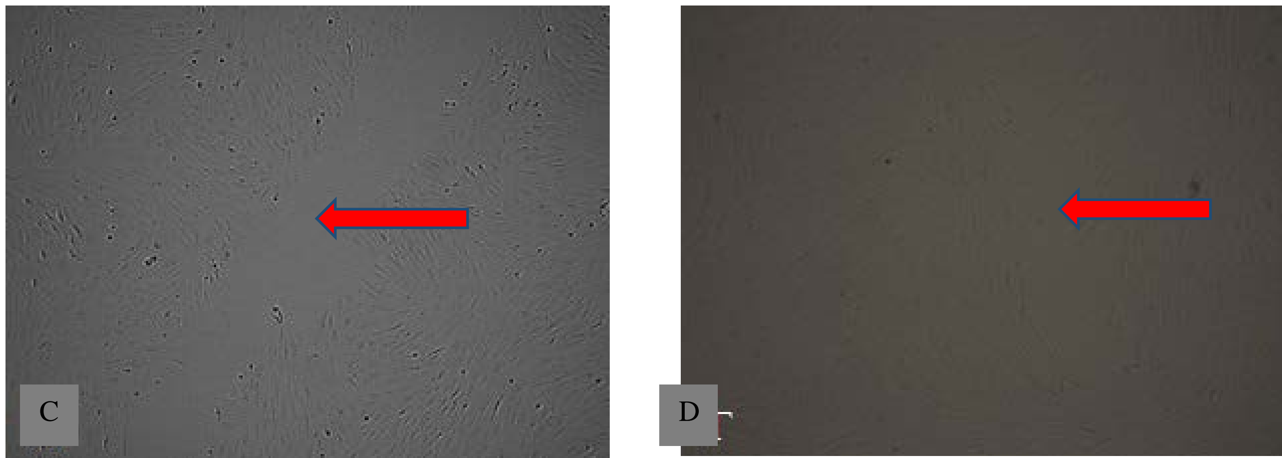


Fig. 2. Wound Healing Assay. 18 hours of cultivation after forming a mark on the single layer formation. A—hMSCs cultivated in standard growth medium, B—hMSCs cultivated in medium containing WoL diluted at the concentration of ×1. C—hMSCs cultivated in a medium containing AE drug at a concentration of ×1. D—hMSCs cultivated in a medium containing AE drug at a concentration of $\times 10^{-5}$. Inc. ×50. The red arrow indicates the mark location.

The pictures show that the observational group cells are actively migrating and completely overlapped the "defect" on the single layer formation. hMSCs that were cultivated in the medium containing WoL, the cells overlapped the defect by 80%, as well as in the medium with the addition of AE at a concentration of $\times 10^{-5}$. Complete overlap of the defect was observed 24 hours after the start of the experiment. When cultivated in a medium containing AE hydroplasma at a concentration of ×1, the cells did not migrate. Instead, they curled up; single dead cells were observed in the growth medium.

Data on secretory activity of the conditioned environment in the observation completely correlate with the literature data (Table 1).

Table 1.

Results of the study of hMSCs secretory activity in observational and experimental groups

	TNFα (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-10 (pg/ml)
Observation	2.4[2.2; 2.85]	71.8[71.7; 74.6]	65.1[61.9; 67.7]	2.8[1.7; 3.4]
WoL × 0.5	3.5[2.1; 3.8]	68.6[62.4; 71.9]	67.1[60.5; 70.4]	1.8[1.3; 2.2]
WoL × 1	4.8[1.8; 5.4]	70.4[58.7; 72.4]	58.7[54.4; 61.1]	1.6[1.1; 2.3]
WoL × 2	1.8[1.7; 2.1]	75.9[75.1; 78.9]	68.1[67.1; 70.6]	1.3[1.1; 1.6]
AE $\times 10^{-5}$	2.3[1.9; 2.6]	70.3[65.3; 78.7]	68.3[63.9; 71.1]	2.5[1.2; 3.1]
AE × 0.5	0	22.5[16.5; 27.6]*	17.3[16.2; 18.5]*	5.8[4.6; 6.7]*
AE × 1	0	19.1[10.5; 25.2]*	15.7[15.3; 16.4]*	6.2[4.9;7.1] *
AE × 2	0	11.8[10.3; 12.8]*	12.9[11.3; 13.8]*	5[4.3; 5.5]*

* significant statistical differences ($p<0.05$). The data is presented by median and quartile. The difference was analysed using Mann-Whitney method.

The secretory activity of hMSCs cultivated in a medium containing WoL and AE in extremely low concentrations is comparable with the observation value. The level of secretion by TNFα cells is slightly higher. No statistically significant differences were found. The secretory activity of IL-6/-8 in hMSCs, cultivated in a medium with the addition of AE in normal concentration, was 5 times lower; TNF-α cells did not secrete, the secretion of IL-10 was 2–3 times higher. Apparently it is due to early cell death.

Conclusions

Two samples of hydroplasma were analysed as part of this paper, WoL and AE. Each sample was analysed using 3 different concentrations; each analysis was repeated 3 times. The average value and standard deviation are calculated from the data obtained. When assessing the overall effect of WoL and AE hydroplasma in extremely low concentrations on the proliferative and secretory activity of hMSCs, no

statistically significant difference was found compared to the hMSCs observational culture. When estimating the total impact of AE hydroplasma, the death of individual hMSCs was observed from the first day of cultivation. By the fifth day after the beginning of the experiment, all the cells were dead. It was associated with a low content of TNFα, IL-6/-8 in the conditioned environment, as well as a minor increase in the amount of IL-10.

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