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Effects of succinic acid on dermal fibroblasts during cultivation under extremely hypoxic conditions

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ABSTRACT

Succinate is one of the most important raw materials in the energy-producing cycle, enabling a 'shortcut' route for ATP production via oxidative metabolism. Fumarate is produced from succinate by the oxygen-dependent succinate dehydrogenase. In this study we investigated the influence of different succinic acid concentrations on the process of cellular senescence under normal and hypoxic conditions. The most promising doses were chosen by using a cell viability test (0.2–1.5 mM). After the long-term cultivation it was shown that there was no significant difference in senescence β-galactosidase expression between samples with variable concentrations of succinate. Despite this fact, large differences in expression levels of senescence and pluripotency markers were found between the samples depending on the oxygen level and concentration of succinic acid.

1. Introduction

During hypoxia succinate can act as an important metabolic intermediate leading to adenosine triphosphate (ATP) generation as a result of a shift from oxidative phosphorylation to glycolysis [1]. While succinate is well known for its role as an intermediary metabolic product in the Krebs cycle, accumulation of succinate in mitochondria due to hypoxia may cause it to diffuse out into the general circulation through a number of putative membrane transporters and may act as a signaling molecule in peripheral tissues [2]. In the cytosol, succinate plays regulatory roles beyond primary metabolism. Elevated cytosolic succinate levels may promote protein post-translational modifications by addition of succinyl groups to lysine residues [3]. In addition, succinate is a critical mediator of the hypoxic response, an important piece of the puzzle regarding tumorigenesis and it is involved in protein succinylation, a newly discovered posttranslational modification [4]. The significant influence of succinic acid on different processes in organism and its role in energy metabolism gives a basis for investigation of succinate in the context of cellular senescence under different oxidation conditions.

2. Materials and methods

Primary fibroblast culture conditions. Primary dermal fibroblast culture was obtained from healthy middle-aged female volunteer donor. The explants were dried and attached to the culture dish, then cultured in complete medium (DMEM enriched with 10% fetal bovine serum), at 100% humidity, 37 °C, with 5% CO_2. Medium change and monitoring were performed three times a week. After fibroblasts formed large colonies, they were seeded in culture flasks t25 and t75 at a concentration of 5000 per 1 cm^2 [5].

MTT-test. To perform the MTT test, cells of the respective lines were seeded on 96-well plates (NEST) in standard culture medium (200 μl/well), at a concentration of 5000 cells/cm2. The range of succinate concentrations corresponded to a series of two-fold dilutions from maximum of 3.12 mM to minimum. The results were measured with spectrometer-calorimeter VARIOSCAN software [6]. The obtained data were organized and processed in Microsoft Excel. Statistical processing included determination of the mean and standard deviation.

Long-term cultivation with different succinic acid concentrations. Cell culture was cultivated in DMEM medium, supplemented with 10% FBS. After the first passage, the total amount of cells was transferred into 8 t25 flasks and cultivated for 15 passages with two different O_2 concentrations (20% O_2 and 1% O_2). All cell cultures were cultivated with different concentrations of succinic acid in the medium (0.2 mM, 1 mM, 1,5 mM), and control flasks didn't contain any succinic acid at all. While passaging, all flasks were seeded simultaneously in a concentration of 5000 per 1 cm^2. After 15 passages, when first signs of senescence were observed, all cells were detached and analysis of pluripotency and senescence markers was performed.

SA-β-galactosidase detection. For detection of SA-β-galactosidase

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activity in cells we used a Senescence Detection Kit (ab65351, Abcam, Cambridge, UK), which is designed for histochemical detection of SA-β-Gal activity in cultured cells and tissue section. Cells positive for SA-β-gal were quantified by counting positive and negative cells in at least five random independent fields. For more proper counting of cells in the microenvironment, after SA- β -galactosidase staining an additional contrast staining with eosin was performed. After development of a blue color, the results of SA-β-galactosidase histochemical detection in cells were observed under a light microscope. The images were analyzed using Ilastik, the cells were counted in ImageJ2 software. The data were statistically preceded in Origin 9.1.

2.1. Quantitative PCR

Cell lysis was performed using the CHAPS reagent [7]. Specifically, each cell pellet was resuspended in an ice-cold CHAPS lysis buffer at a concentration of 100–200 cells μl-1 and incubated on ice for 30 min. DNA extraction was performed using a phenol-chloroform method [8]. Further processing was carried out using DNAse enzyme (Syntol), and reverse transcription (RT) reaction was performed using a commercial Reverta-L kit (AmpliSens) according to the manufacturers' manuals.

Expression of the next human genes was measured with quantitative polymerase chain reaction (qPCR): *p16, DPP4, cMYC, Oct4, Sox2, Lin28,* and *Rps18* was used as a house-keeping gene. Reaction master mix preparation included the following reagents: commercial reagent kit for qPCR (2.5x Reaction mix for the qPCR with SYBR Green I, Syntol), betaine (at 1 M concentration, Sigma-Aldrich) and pairs of specific primers. All samples were tested in triplets, and for a calibration curve, four serial dilutions varying from 1 to 1/81st were made.

3. Results

3.1. MTT test

MTT assay is commonly used for evaluating cell viability and cytotoxicity of potentially hazardous substances. We used this assay to assess potential cytotoxicity of succinic acid and to choose the most promising concentrations for subsequent studies of geroprotective properties of succinic acid. The results of the MTT test are presented as the percentage of viable fibroblasts in the presence of succinate at different concentrations (Fig. 1). It is evident that high concentrations of succinic acid (12.5 mM, 25 mM and 50 mM) were highly toxic in dose dependent manner. But in lower concentration (0.2 mM, 0.39 mM, 0.78 mM and 1.55 mM) succinate wasn't resulting to the cell toxicity and the level of cell proliferation was comparable to the level of control fibroblasts untreated with succinic acid.

3.2. Long-term cultivation with different succinic acid concentration

Within 15 cultivation passages, cell lines gain first features of senescence: fibroblasts change their morphology from a spindle shaped to an enlarged, flattened and irregular; time of cell doubling becomes prolonged; during passaging the quantity of detached cells decreases. These features are more typical for the cultures cultivated in low O_2 atmosphere (1% O_2) and with higher concentrations of succinic acid (1 mM, 1.5 mM). When the cultures showed the first signs of aging SA-β-galactosidase (Fig. 2) and PCR tests were performed.

3.3. SA-β-galactosidase detection

First SA-β-gal staining was performed at the beginning of long-term cultivation, when no signs of senescence were yet observed (data not shown). All cultures had minimal amounts of SA-β-gal positive cells (<2%) with no visual or statistically significant difference between the samples.

After 15 passages, final staining was performed (Fig. 3). In the groups with low level of atmosphere O_2 more SA-β-gal positive cells were detected in the samples with higher concentrations of succinic acid. But the statistical analysis did not show any significant difference. In a group with higher level of atmosphere O_2 there was no big difference between samples analyzed visually or statistically.

3.4. Analysis of senescence markers expression

Different levels of senescence markers expression were observed in the samples with various cultivation conditions (Fig. 4).

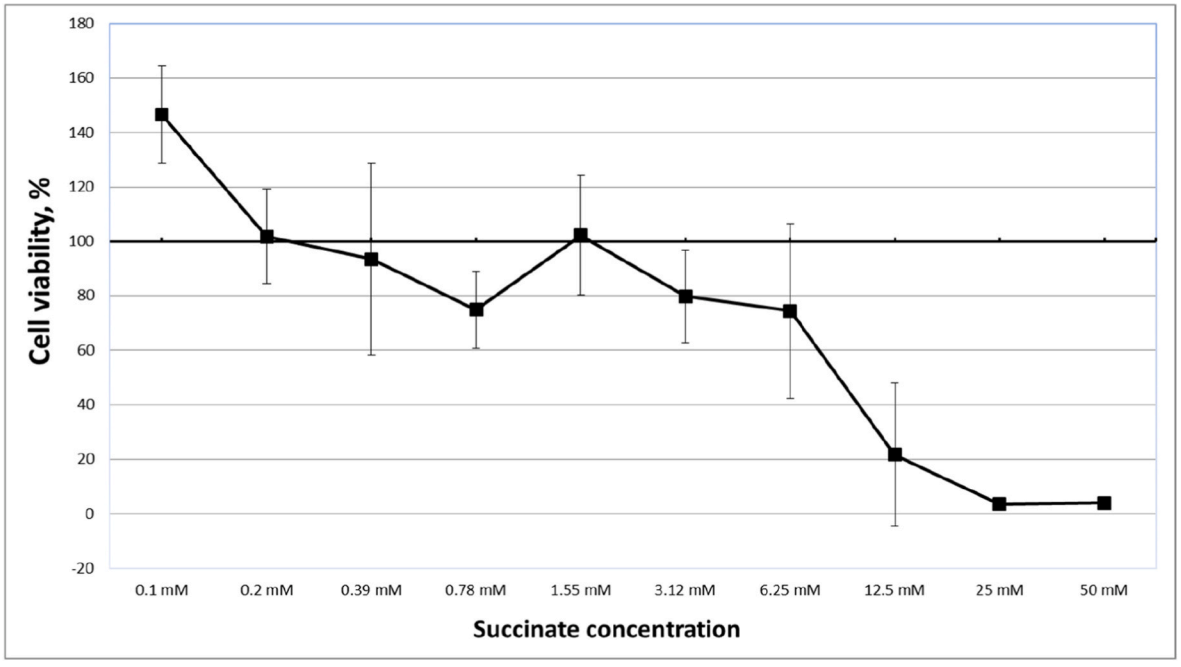

Fig. 1. Percentage of the cell viability (MTT test) deviation in fibroblast cell cultures treated with different concentrations of succinic acid compared to the control. The control level is indicated by the horizontal dashed line..

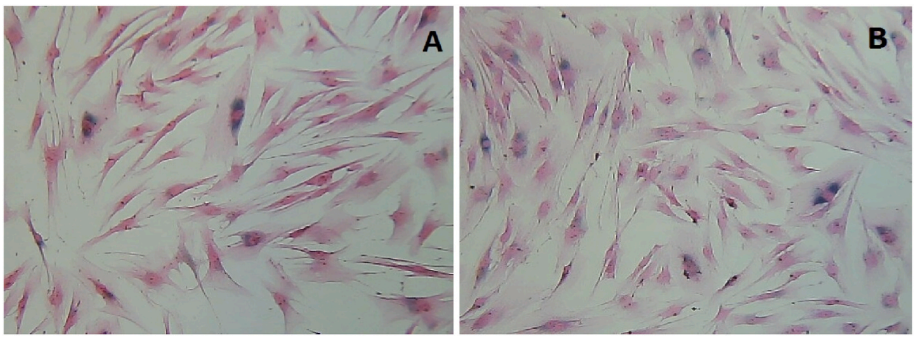

Fig. 2. Control fibroblasts (A) and fibroblasts cultivated with 1,5 mM of succinate (B) at low O_2. The cells were fixed and stained for SA-β-galactosidase detection with additional contrasting eosin staining.

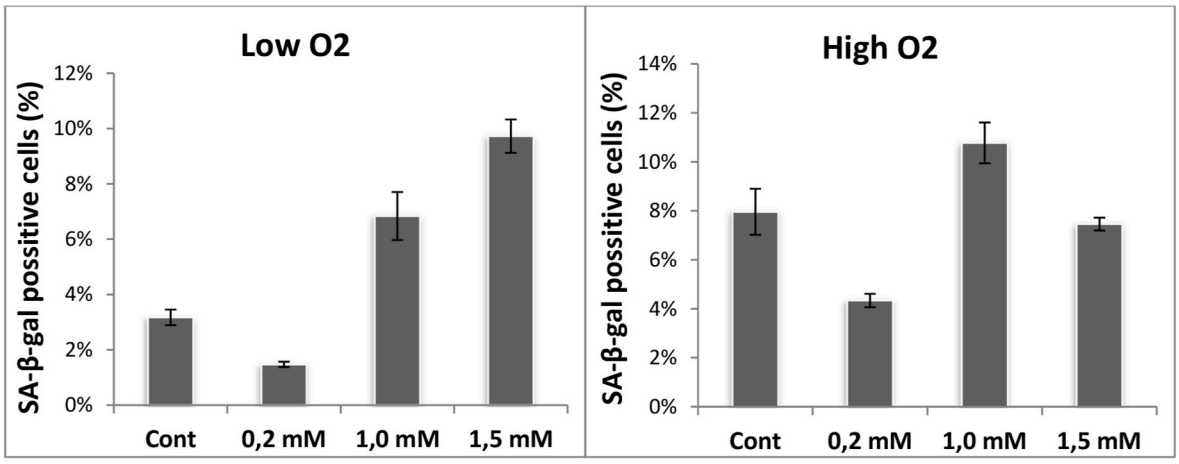

Fig. 3. Percentage of SA-β-gal positive cells cultivated under different O_2 conditions ("Low O_2" – 1% O_2; "High O_2" – 20% O_2), with different succinate concentrations (0.2 mM, 1.0 mM, 1.5 mM) and without it (Control group). Values are expressed as a percentage of positive from all samples ± SD (n = 5). Tested and control groups were compared using Mann–Whitney *U* test and no significant difference was observed.

Fig. 4. Senescence markers expression of in-vitro aging dermal fibroblasts, cultivated under different O_2 conditions ("Low O_2" – 1% O_2; "High O_2" – 20% O_2) and with different concentrations of succinic acid (0.2 mM, 1.0 mM, 1.5 mM) or without it (Control group). The statistical analysis was performed using Kruskal–Wallis H-test, significant difference between samples was observed for all markers (p = 0.02).

High expression levels of p16 were observed after 15 passages of cultivation in the samples without succinic acid and with its low concentrations (0.2 mM). Extremely high expression levels were detected in the samples from the control group, cultivated under low O_2 conditions. On the other hand, the levels of this senescence marker were becoming lower in the samples with higher concentrations of succinate both in high and low O_2 conditions.

For DPP4 there was a big difference in expression levels between the samples under the high and low O_2 conditions. The samples cultivated in the high O2 atmosphere showed the much higher expression levels of DPP4, than the ones cultivated under hypoxic conditions. Moreover, there were pretty low levels of DPP4 detected in the control groups and,

much higher levels of it, was detected in the groups with the least concentration of succinic acid. In the groups with 1 mM and 1.5 mM of succinate, the decrease of DPP4 expression was observed.

3.5. Analysis of pluripotency markers expression

The large difference in expression of pluripotency markers was detected between the samples with various conditions of cultivation (Fig. 5).

Very high levels of Sox2 and cMYC expression were detected in the cultures with low O_2 and 1.5 mM of succinic acid, compared to the samples under all other conditions.

For Oct4 and Lin28 markers the expression pattern of expression was very similar to Sox2 and cMYC, but except high expression in samples with low O_2 and 1.5 mM of succinate, significant level was detected in samples with high O_2 and 1.5 mM of succinic acid in medium.

4. Discussion

Firstly the most promising concentrations of succinic acid in culture media were chosen. During the MTT test it was clear that succinate in all working concentrations did not result in beneficial effects on proliferation activity of human dermal fibroblasts culture during short-term cultivation (3 days). But low concentrations were chosen for further experiments after induction of cellular senescence by the method of gradual cultivation with cell passaging [9].

Despite the first visual signs of senescence in all samples after long-term cultivation (15 passages), the percent of SA- β-galactosidase positive cells in all samples was not high, and it is interesting that no statistically significant difference between samples was observed. More senescent cells were found in samples with high concentration of succinic acid at low O_2 conditions, but such tendency was not observed in the group at high O2. These results may indicate that higher levels of oxygen in atmosphere could have some geroprotective properties for human fibroblast culture.

The most promising results were observed in the experiments for pluripotency and senescence markers expression. Positive tendency in lower senescence markers expression in the samples with higher concentration of succinic acid demonstrate that succinate can in some way regulate the cellular metabolism. In the long run it may reduce the expression of some senescence markers and possibly slow down the cellular aging. But it is important that according to our results, not all signs of aging showed statistically significant improvement in the samples with high succinate content, which means that further studies are needed to identify and confirm the geroprotective properties of succinic acid.

It is interesting that very high expression levels of pluripotency markers were observed in the samples with high O_2 concentration and 1.5 mM of succinate. It is possible that in this case succinate supplement was acting like the inducer of pseudohypoxia for human dermal fibroblasts culture, inducing metabolic reprogramming from oxidative phosphorylation to glycolysis under the normal oxygen condition. On

Fig. 5. The pluripotency markers expression in aging dermal fibroblasts, cultivated under different O_2 conditions ("Low O_2" – 1% O_2; "High O_2" – 20% O_2), and with different concentrations of succinic acid (0.2 mM, 1.0 mM, 1.5 mM) and without it (Control group). The statistical analysis was performed using Kruskal–Wallis H-test, significant difference between samples was observed for all markers ($p = 0.02$).

the other hand, it is possible that the same concentrations of succinic acid did not affect the samples under the real hypoxic conditions in the same manner because of naturally produced amounts of succinate in the medium. Combined with added succinate it could lead to its too high concentrations and cause an inhibitory effect on the cell cultures. It was shown by Mao et al. that the state of pseudohypoxia promoted cell proliferation, migration, and might help maintain stemness of human periodontal ligament cells in vitro [10]. In our study we detected a great increase in the expression of pluripotency markers, which may be an evidence of the succinate ability to improve differentiation capability in primary cell cultures. Thus, further research is needed to study the succinate ability to maintain cell potency to differentiate.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101429.

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