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Exploring Senescence Reversal Potential of Antioxidants in Human Foreskin Fibroblasts

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Cell aging (senescence) is a process of irreversible blocking of the cell cycle and inability to perform normal replication. By blocking cell division, aging prevents the proliferation of damaged old cells and makes them resistant to apoptosis. In addition to reduced proliferative potential, aging cells are characterized by specific morphology, increased β-galactosidase activity, and changes in some important signaling pathways such as p53/p21. Aging cells have a typical secretory phenotype called SASP. Aging can be divided into two categories based on the mechanism involved – replicative aging and stress-induced aging. The latter is caused by accumulation of oxidative stress. The aim is to study the effectiveness of several antioxidants – resveratrol, quercetin, and vitamin C to slow down cell aging caused in cell culture of primary human foreskin fibroblasts (HFF), exposed to oxidative stress by treatment with high (25 mM) concentrations of glucose.

Key words: senescence, oxidative stress, glucose, p53, quercetin

Introduction

Senescence is an innate state of the cell. Often, it is the result of chronic stress exposure and is an alternative to the standard fate that is apoptosis. Cells opt for senescence to avoid potential oncogenesis, however by entering this state, in the long term they start to exude a plethora of pro-inflammatory molecules which is referred to as the SASP (senescence associated secretory phenotype). Accumulation of senile cells, emitting SASP elements throughout the life of an organism can lead to health complications. *In vitro* cultivated cells that have entered senescence exhibit a specific morphology characterized by flattening and enlargement of the cell. Additionally, a marker for such cells is the enzyme senescence-associated β-galactosidase (SA-β-gal) which is hardly expressed in any type of normal cell [5]. Even though ROS have a designated role, a

lack of balance between them and antioxidant systems results in their surplus which could lead to senescence. Evidence suggests that supraphysiological concentrations of glucose have the potential to generate ROS. Reactive oxygen species can influence the expression of GLUT transporters, their vesicular transport, as well as their behavior in the membrane. On the other hand, GLUT transporters mediate the transport of dehydroascorbic acid (DHA) into the cell and mitochondria, and it is an important tool in maintaining the balance of free radicals in the cell [7].

Materials and Methods

Cultivation

Primary human skin fibroblasts (Human Skin Fibroblasts, HSF) from the collection of the Department of Cytology, Histology and Embryology are used. Routinely, cells are grown in low glucose medium supplemented with 10% FBS/FCS and antibiotics. They were subsequently planted in 10 cm diameter petri dishes with low glucose medium (LG) as in physiological conditions (5.5 mM), high glucose (25 mM – HG) to mimic a diabetic state and low glucose, but with added artificial sweetener mannitol at a concentration of 25 mM (MN), comparable to the high glucose medium. They are grown under standard conditions – temperature 37°C and 5% CO_2. Every day, the medium of the cells is replaced with a new one. The experiment was conducted in a six-well plate where cells are inoculated at 5×10^4 concentration.

Staining

A β-galactosidase stain was performed to quantitively determine senescence where fixed cells from five consecutive days were incubated with the substrate X-gal which upon metabolization by SA-β-gal positive cells yields a blue-green color. HFF were cultivated in medium with 5,5 mM (control/LG), 25 mM glucose (HG) and 5,5 mM glucose + 25 mM mannitol (MN). Culture medium was removed, cells were washed with PBS followed by fixation with 3% formaldehyde for 5 min at room temperature. A freshly prepared staining solution is then added, and the plates or petri dishes are incubated for 24 h in the dark at a temperature of 37 °C. The development of staining was observed using an inverted microscope or binocular magnifier. Once sufficiently intense staining was obtained in the LG-grown control cells, the solution was removed, and the wells were rinsed three times with PBS and the plates were stored in a refrigerator at 4 °C.

Expression analysis

A Western blot analysis was conducted to evaluate the expression levels of p53 and caveolin-1. The transcription factor p53 plays a key role in the fate of a stressed cell – apoptosis, senescence, or carcinogenesis. Precast 8-16% Mini-PROTEAN TGX Stain-Free Gel gradient gel is used. Electrophoresis was carried out for 30 min at 200 V DC voltage. For the transfer of the electrophoretically separated proteins from the gel to the nitrocellulose membrane, a Trans-Blot Turbo Transfer Pack (BIORAD) and the Trans-Blot® Turbo™ Transfer System of the same company were used. The manufacturer's instructions were followed.

Once the proteins have been transferred, they should be treated with 5% dry milk dissolved in TBST for 30 min. Upon blockage, the membrane was treated with the first antibody (Upstate Biotechnology anti-P53, Santa Cruz rabbit anti-caveolin 1, Santa Cruz mouse anti-GAPDH) at 4°C for 24 hours. After washing three times with TBST to wash off the unbound first antibody, it was incubated with a second antibody for 1 h, anti-mouse and anti-rabbit, respectively, which are conjugated with peroxidase (HRP). This is followed by a final triple wash for 15 min each with TBST. For development, the membrane was incubated in the dark for 1 min with Clarity™ Western ECL Substrate. After development, the membranes were viewed on a ChemiDoc® Imaging System (BIORAD). ImageJ program was used for data analysis.

Visualization of ROS and lipid peroxidation

For visual assessment of the damage by ROS and to confirm its abundance in cells, exposed to higher glucose levels immunofluorescent microscopy methods were used. For quantative measurement of ROS the DCFDA / H2DCFDA – Cellular ROS Assay Kit was used. For the purposes of the experiment, cells were grown on glass coverslips under standard conditions. Cells were washed once or twice with 1x buffer provided by the manufacturer and stained with diluted DCFDA solution. Incubate for 45 min in the dark at 37 °C. Wash again once or twice with 1x buffer. Live cells were mounted in Mowiol fluorescence microscopy medium (Sigma-Aldrich) and observed under a fluorescence microscope.

To assess the damage ROS inflicted on the cells was utilized the Image-iT® Lipid Peroxidation Kit. This methodology allows indirect observation of ROS because the active ingredient in the kit changes color when oxidized from red to green. For this purpose, cells are seeded on glass coverslips in medium with a low and high concentration of glucose and incubated overnight at 37°C. Add Image-iT Lipid Peroxidation Sensor in medium or buffer with a final concentration of 10 μM and incubate for 30 min at 37°C. The medium was removed and the cells were washed three times with PBS. It was embedded in Mowiol (Sigma-Aldrich) medium and observed under a fluorescence microscope.

Senolytic potential assessment

An experiment to explore the senescence reversal potential of different substances was designed. We tested the antioxidants Vit C 100 μM, resveratrol 10 μM, quercetin 5 μM and the senolytic and approved drug metformin 500 μM all of which were added to 25 mM glucose medium to establish whether the generated ROS by the high glucose would be neutralized by the antioxidants. In three six-well plates, 0.5×10^5 cells were seeded per well.

For five days, the medium is changed daily, preferably at the same time. The following concentrations of antioxidants are used – 100 μM vitamin C, 5 μM quercetin, 10 μM resveratrol and 500 μM metformin. The respective antioxidant was pre-added to 25 mM glucose medium. After five days, the steps to stain for β-galactosidase were followed, ending with counting and quantification of the stained cells as described above.

Statistical analysis

For statistical analysis Student's t-test was applied with significance at $P < 0.05$.

Results and Discussion

Staining

Generated data (**Fig. 1**) display a time-dependent increase in the percentage of senescent cells compared to all cells in the group treated with 25 mM glucose and no changes in other groups (5,5 mM and 5,5 mM glucose + 25 mM mannitol). The data resonate with the preliminary results of teams such as Wang et al., 2019 [8] and Ha & Lee, 2000 [4] and provide basis for further exploring the suspected connection between glucose and senescence.

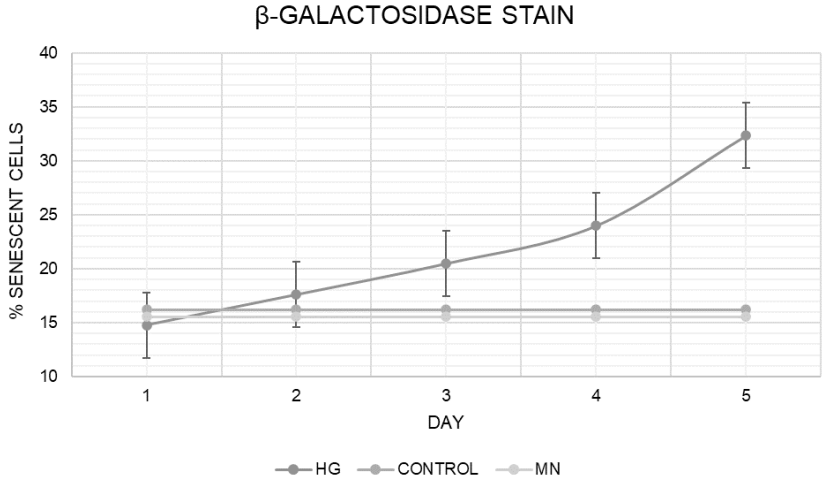

Fig.1. Time-dependent increase in the percentage of senescent cells after cultivation of human skin fibroblasts with 5,5 mM (control-LG), 25 mM glucose (HG) and 5,5 mM glucose + 25 mM mannitol (MN).

Expression analysis

Considering the typical morphological changes of senescent cells caveolin 1, a crucial membrane protein is a logical target for monitoring during those changes. Results from the experiment (**Fig. 2**) point toward a statistical rise in the expression of both molecules – caveolin 1 and p53. This provides a clear link between p53 and cellular aging and makes the molecule an object of interest for further study as well as a target for antiaging drugs. The data obtained are consistent with Atadja et al., 1995 [1], showing a similar increase in p53. Apart from that results open the possibility to use caveolin 1 not only as a marker for various cancers [3] and as a marker to detect senescent cells, similar to SA-β-gal.

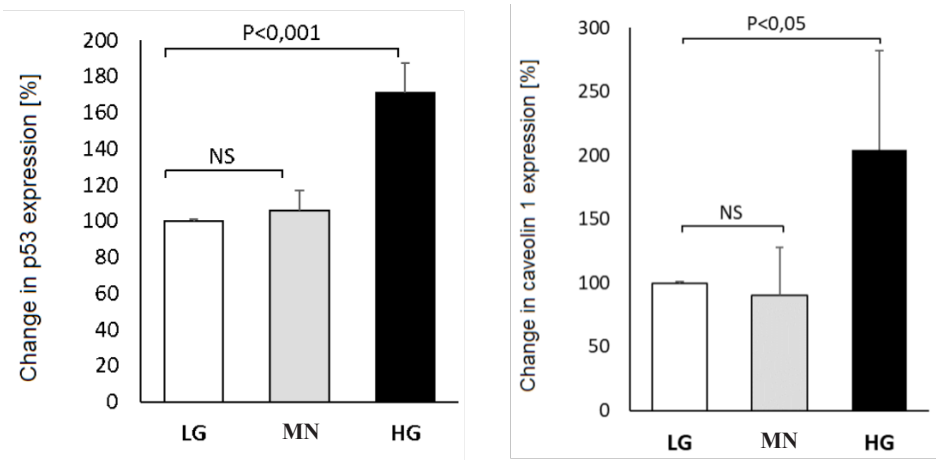

Fig. 2. Expression of p53 and calvelolin in cultivated human skin fibroblasts with with 5,5 mM (control-LG), 25 mM glucose (HG) and 5,5 mM glucose + 25 mM mannitol (MN).

Visualization of ROS and lipid peroxidation

The results in **Fig. 3** show a clear increase of fluorescence in the cells cultivated in 25 mM glucose. These data are in sync with those obtained by Buranasin et al., 2018 [2] demonstrating that exposure to high levels of glucose in human gingival fibroblasts leads to an increase in ROS levels in the cells. The produced images in **Fig. 4** depict that in the 5,5 mM group (LG) the red (showing all lipids in the cell) and green (showing oxidized lipids) channels have similar intensity. On the other hand, the higher glucose group depicts noticeable increase in intensity at the green channel corresponding to higher levels of lipid oxidation hence ROS. Similar experiment investigating the effect of high glucose on LDL oxidation found that treatment with 25 mM glucose (HG) induced increased levels of oxidized LDL. Although these are lipoprotein complexes, the data match with ours because in both cases we are talking about HSFs treated with 25 mM glucose [6].

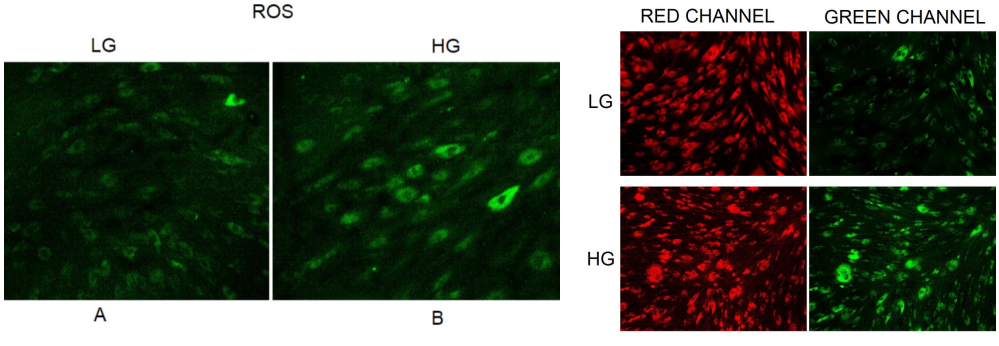

Fig. 3 and **Fig. 4.** Fluorescent images of cells cultivated human skin fibroblasts with 5,5 mM glucose (LG) or 25 mM glucose (HG)

Senolytic potential assessment

Initially, we explored the effect of treatments with antioxidants (Vit. C, quercetin-Q, resveratrol-Res and metformin-Met) on the levels of senescence through β-galactosidase stain (**Fig. 5**). After the treatments, Western blot analysis (**Fig. 6**) was repeated to unveil whether treatment with the explored substances affected the expression of p53 and caveolin 1. Both experiments point toward a significant reduction in the percentage of senescent cells, as well as the expression of caveolin 1 and p53. Quercetin (Q) appears to have to the most noticeable senolytic properties.

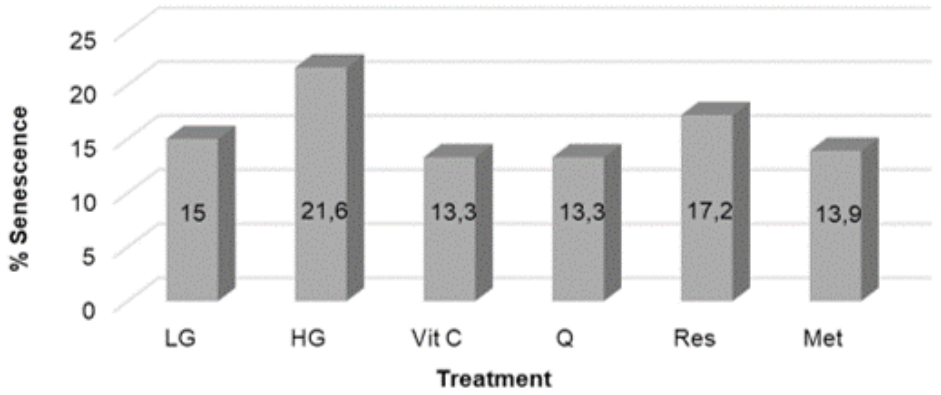

Fig. 5. Effect of treatments with antioxidants (Vit. C, quercetin, resveratrol and metformin) on the levels of senescence (through β-galactosidase stain) of human skin fibroblasts, cultivated with 25 mM glucose (HG). LG – low glucose (5,5 mM)

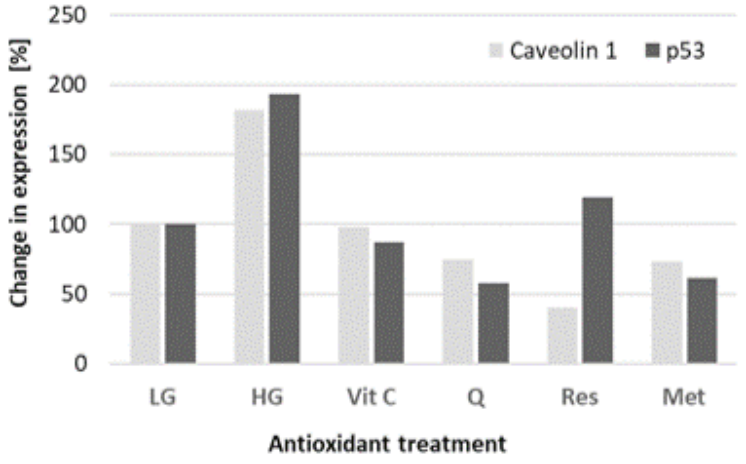

Fig.6. Effect of treatments with antioxidants (Vit. C, quercetin, resveratrol and metformin) on the expression of caveolin 1 and p53 in human skin fibroblasts cultivated with 25 mM glucose (HG). LG – low glucose (5,5 mM)

Conclusions

Based on the obtained results, the following conclusions can be suggested:

1. Cultivation of human skin fibroblasts in high glucose medium (25 mM) causes an increase in the percentage of senescent cells expressing β-galactosidase and this effect is not due to increased osmotic pressure of the medium.

2. High glucose induces an increase in the expression of the cellular senescence markers caveolin 1 and the transcription factor p53. A similar effect was not observed in control cells grown in medium with increased osmotic pressure (5.5 mM glucose + 25 mM mannitol).

3. Hyperglycemia causes an increase in cellular levels of reactive oxygen species (ROS) and lipid peroxidation.

4. The addition of antioxidants vitamin C (100 μM), quercetin (5 μM), resveratrol (10 μM) and the senolytic metformin (500 μM) effectively reduced senescent cells to and even below control levels, supporting the hypothesis that hyperglycemia induces cellular senescence by stimulating the production of reactive oxygen species (ROS).

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