In vitro assessment of copper capacity to induce cellular senescence and the potential protective role of α-MSH

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Background

- Cellular senescence is associated with the aging process. This cell fate can be induced by various stimuli, even by elements indispensable to life, such as copper (Cu), through the creation of reactive oxygen species.
- Obesity is seen as a form of premature aging, where increased blood Cu levels and accumulation of senescent cells in the adipose tissue are observed.
- Consequently, drugs that ameliorate obesity may also have systemic benefits, delaying aging itself. The melanocyte-stimulating hormone (α-MSH) is one of these examples, since α-MSH-treated obese mice had reversal of various features described in obesity (1).

Aim of the project

- Analyse if the high concentration of copper seen in obesity can be one of the inducers of the formation of senescent cells and examine if compounds that ameliorate obesity can prevent the formation of senescence.

Methodology

1. Cytotoxicity analysis

- Gradient of [CuSO4] 3 hours for 3 days in DMEM Neutral red assay
- Cytotoxicity analyses

In 96 well plates, 20 thousand 3T3-L1 pre-adipocytes were plated per well and incubated with several concentrations of CuSO4 (250, 500, 750, 1000, 1250 and 1500 μM), for 3 hours during 3 days in DMEM. After the stimuli, the cells were washed with 150 μL of PBS and incubated with 200 μL of a full media supplemented with 1% of neutral red for 2h in the cell culture incubator. Then, wells were washed again with 150 μL of PBS and the cells were lysed with 150 μL of “dissolution solution” 1% acetic acid + 50% of ethanol. The coloration obtained was measured at 540 nm in a microtiter plate reader spectrophotometer.

2. Senescence phenotype

- [CuSO4] 750 μM 3 hours for 3 days in DMEM
- [Na2SO4] 750 μM
- [CuSO4] 750 μM + α-MSH 1 μM

2a) Proliferation capacity

In 24 well plates, 20 or 40 thousand 3T3-L1 pre-adipocytes were plated per well and incubated with 750 μM of Na2SO4 or CuSO4, respectively, for 3 hours during 3 days in DMEM medium and rested until the 6th day. Additionally, in the same conditions as the 750 μM of CuSO4 induction, cells were incubated with 1 μM of α-MSH, that was replenished every day. Cells were counted from the 3rd to the 6th day and the values normalized for the third day.

2b) Senescence markers

The protocol described in the “proliferation capacity” section was carried out in 6 well plates with 150 thousand cells/well. At the 6th day, RNA extraction and purification was performed using the trizol reagent directRNA Kit (grisp) following the manufacturer’s protocol. RNA concentration and purity was assessed with Nanodrop and 1μg of RNA was used for cDNA synthesis, with the N2P First-Strand cDNA Synthesis Kit (Invitrogen). Real time PCR were carried out in the StepOne Real-Time PCR System (Applied Biosystems) using SYBR® Select Master Mix (Life Technologies) and specific primers for p21, p16, MMP3 and TRF (telomere lengthening gene).

2c) SA-β-gal expression

Using the same procedure described in b), at the 3rd day cells were trypsinized to a new 6 well plate with 10 or 30 thousand cells per well depending on whether the stimulation was by Na2SO4 or CuSO4, respectively. At the 6th day cells were washed twice with 1mL of PBS and fixed for 5 min with 2% formaldehyde + 0.2% glutaraldehyde in PBS. Fixed cells were then incubated for 20h at 37ºC with coloration solution shown in Tahaya protocol. (2) After coloration the wells were washed with PBS and a final fixation with methanol was done. Cells were left to dry in the dark until counting.

Results and Discussion

1. Cytotoxicity analysis

750 μM CuSO4 was the highest Cu concentration evaluated that did not significantly induced cell cytotoxicity, when compared to controls.

2. Senescence phenotype

a) Proliferation capacity

3T3-L1 cells treated with CuSO4 showed a reduced growth capacity, while α-MSH did not attenuated this proliferation decrease. Cells incubated with the control Na2SO4 remained proliferative.

No significant differences were found for markers of senescence (p16, p21 and MMP3) with CuSO4 stimuli or α-MSH treatment.

A higher number of SA-β-gal positive cells were seen for cells incubated with CuSO4 while α-MSH did not decrease the appearance of this senescence marker.

Conclusions

• The results obtained show that 3T3-L1 cells incubation with CuSO4 leads to:

1. Attenuation of cell proliferation
2. Higher number of SA-β-gal positive cells.

• More characteristics should be evaluated and remeasured to predict if CuSO4 can induce senescence in 3T3-L1 cells and if α-MSH can protect these cells from the appearance of this phenotype.

Literature