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SUPPLEMENTARY MATERIAL

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Low ozone concentrations promote adipogenesis in human adipose-derived adult stem cells

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Materials and Methods

Isolation of primary cells and in vitro cell culture

Subcutaneous adipose tissue samples were harvested from three healthy women, 30-45 years old, subjected to ambulatory liposuction from the medial area of the knee for aesthetic purposes. Liposuction procedure was performed after informed consent according to Sbarbati *et al.*[1]

For hADAS cells isolation, 10 mL of subcutaneous adipose tissue were placed in a sterile 50 mL polypropilene tube and incubated for 45 min at 37°C with 20 mL solution containing 0.2g type I collagenase and 2% (w/v) bovine albumin serum. After digestion, the samples were centrifuged at 400 g for 5 min at room temperature (RT). The pelleted stromal vascular fraction was suspended in 160 mM NH_4Cl in phosphate buffer saline (PBS) to lyse blood cells, filtered (pore size 0.45 µm) and centrifuged at 200 g for 10 min at RT. The resulting pellet was re-suspended in 6 mL DMEM/F12 medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) supplemented with GlutaMAX™, 10% (v/v) fetal bovine serum, 0.5% (v/v) amphotericin B, 100 units/mL of penicillin and 100 µg/mL of streptomycin (all products from Gibco by Life Technologies), seeded in a 25 cm^2 flask (FalconTM BD Medical) and incubated at 37°C in 5% CO_2 humidified atmosphere for 72 h.

Cells were trypsinized (0.25% trypsin in PBS containing 0.05% EDTA) when sub-confluent, and seeded on glass coverslips in 6-multi-well plastic microplates ($5x10^3$ cells per well) for light and TEM analyses. For differentiation into the adipoblastic lineage, 24 h after seeding, the growth medium was substituted with the differentiation medium containing 0.2 mM isobutylmathylxanthine, 1 µM dexamethasone, 10 µM rosiglitazone, and 10 µM insulin (all products from Sigma-Aldrich). hADAS cells were grown for 16 days in differentiation medium changed every 3 days and subsequently maintained for further 4 days in DMEM/F12 medium supplemented with 10 µM insulin.

In order to evaluate the possible role of adipogenic factors on the effectiveness of mild ozonisation, gas treatments and lipid measurements were performed also in hADAS grown in DMEM/F12 medium without isobutylmathylxanthine, dexamethasone, rosiglitazone and insulin.

Cell treatment with O_3

According to our ozonisation protocol,[2] hADAS cells adhering to glass coverslips were exposed to O_2-O_3 gas mixtures with O_3 concentrations (5, 10 and 20 µg O_3/mL O_2) successfully used for both systemic and topical treatments.[3] The gas was produced by an OZO2 FUTURA apparatus (Alnitec s.r.l.), which generates O_3 from medical-grade oxygen, and allows the photometric real-time control of gas flow rate and O_3 concentration. Cells exposed to pure O_2 were used to discriminate the effect of O_3, while cells exposed to air served as control. Cells were treated at early (6 days), intermediate (16 days) and late (20 days) differentiation steps and the effects were evaluated 2 h and 24 h after gas exposure.

Cell viability

After 6 days in differentiation medium, hADAS cells were exposed to gas treatment. The fraction of dead cells was estimated by the Trypan blue test after 2 h and 24 h from the treatment. Cells were detached by mild trypsinization and stained in suspension for 2 min with 0.1% Trypan blue in the culture medium: cells permeable to the dye were considered as non-viable and their percentage was estimated by microscope counting on a Burker hemocytometer. Data were expressed as the mean of three independent experiments ± standard error (SE).

Since 20 µg O_3/mL was found to significantly increase cell death (Figure 1), this concentration was excluded.

Oil Red O staining

Oil Red O staining, a dye suitable for neutral lipids and cholesterol ester, was used to visualize lipid droplets (LDs) inside hADAS cells. The cells were processed for light microscopy 2 h and 24 h after treatment: they were washed in PBS, fixed with 4% (v/v) paraformaldehyde in PBS, pH 7.4, for 15 min at RT, rinsed, incubated with Oil Red O (Bio-Optica) for 20 min at RT, washed, counterstained with Mayer's Hematoxylin ready to use solution (Bio-Optica) for 1 min at RT, washed and finally mounted in 1:1 mixture of glycerol:PBS.

Morphometric and statistical analysis

hADAS cells were observed with an Olympus BX51 equipped with an QICAM Fast 1394 digital camera (QImaging). Four randomly selected fields per experimental conditions were imaged at 40X, and the cells (n=37±9) were analysed by using ImageJ software: cytoplasm and individual LD areas were measured, and the percentage of cytoplasmic area covered by LDs was calculated as an indicator of the total lipid content independently from the cell size.

hADAS cell population grown in the absence of adipogenic factors showed only a few clones differentiating into the adipoblastic lineage. For evaluating the effect of mild ozonisation on adipogenesis, only cells (n=15) from these clones were considered for the measurements.

The mean values ±SE of LD area and lipid percentage were calculated for each experimental condition. Statistical comparisons between control and treated samples were performed by using Mann-Whitney pairwise test. In addition, the probability density function (Kernel density estimation) was evaluated in order to show the LDs size distribution.[4,5]

Ultrastructural morphology

24 h after treatment, hADAS cells were processed for TEM. At each incubation time, cells were fixed with 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M phosphate

buffer, pH 7.4, at 4°C for 2 h, post-fixed with 1% OsO_4 and 1.5% potassium ferrocyanide at room temperature for 1 h, dehydrated with acetone and embedded in Epon. Ultrathin sections were stained with UAR-EMS Uranyl acetate replacement stain (Electron Microscopy Science), and then observed in a Philips Morgagni transmission electron microscope (FEI Company Italia Srl), operating at 80 kV and equipped with a Megaview II camera for digital image acquisition.

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