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Differential Response of Human Embryonic Stem and Somatic Cells to Non-Cytotoxic Hydrogen Peroxide Exposure: An Attempt to Model *In Vitro* the Effects of Oxidative Stress on the Early Embryo

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Abstract

Human Embryonic Stem Cells (hESCs) potentially offer a unique *in vitro* model to study how an adverse environment during the early developmental stages post-fertilization can affect the physiology of the undifferentiated embryonic stem cells existing in the early embryo and predispose to long term effects on the offspring, according to the Developmental Origins of Health and Disease (DOHaD) concept. A number of unfavourable conditions can affect the development of the early embryo inducing oxidative stress both *in vivo*, for instance in gestational diabetes and *in vitro*, when embryos are derived from Assisted Reproductive Technologies (ART). Therefore, the aim of this study was the development of a novel *in vitro* model to analyse the effects of oxidative stress and the antioxidant response against Reactive Oxygen Species (ROS) in embryonic stem cells in comparison with somatic cells, fibroblasts and endothelial cells. To this purpose we designed an *in vitro* protocol based on hydrogen peroxide (H_2O_2) treatment of 72 h, in order to better resemble the period of embryonic development from the early cleavages to the blastocyst stage. We demonstrate that H_2O_2 treatment induces the modification of crucial oxidative stress biomarkers like ROS and lipid peroxidation levels, and mobilizes several antioxidant enzymes through NFkβ translocation. Moreover we show differences between somatic and embryonic cells in their antioxidant response towards H_2O_2 induced damage. Therefore this study presents a promising *in vitro* model to investigate the effects of oxidative stress conditions on early human embryonic cells.

Keywords: Oxidative stress; Human embryonic stem cells; Reactive oxygen species; Lipid peroxidation; Gene expression

Introduction

Reactive Oxygen Species (ROS) play normal physiological roles such as second messengers of normal cellular signalling; however, when their production exceeds antioxidant cellular defences, this unbalanced redox status is known as oxidative stress [1]. It is now well established that ROS excess is directly implicated in more than 100 diseases [2] and this oxidation process can result in lipid cell-membrane damage, protein modification and nucleic acids mutation [3], playing an important role in the pathogenicity of ageing [4], diabetes [5], cardiovascular [6] and neurodegenerative diseases [7,8]. In this study we focus on the consequence of oxidative stress in a cell culture model comparing human embryonic stem cells (hESCs) with somatic cells in order to investigate the different responses in terms of viability, ROS production, lipid peroxidation and gene expression changes.

In general, ROS are more reactive oxygen species compared to free oxygen, and comprise hydrogen peroxide (H_2O_2), hydroxyl radical (OH-), superoxide radical (O2•-), singlet oxygen and nitric oxide [9]. The different effects of each member of the ROS-family member are determined by the subcellular source, location and duration of these molecules inside the cell [10]. H_2O_2 is a physiological constituent of living cells, involved in signalling mechanisms and continuously produced via diverse cellular pathways. Under physiological conditions it is relatively stable and less reactive compared to other ROS species and it is able to perform a number of rather specific chemical reactions; moreover, it can react with partially reduced transition metals such as Fe^{2+} or Cu^{2+}, generating the highly reactive OH- that increases the oxidative damage in the cell [11]. This causes an overload of electrons in the mitochondrial matrix by which cellular oxygen is reduced to $O_2^{\bullet-}$, which is subsequently dismutated to H_2O_2, generating ROS and damaging the mitochondria [12]. H_2O_2 intracellular concentration is tightly controlled by various enzymatic and non-enzymatic antioxidant systems and it is assumed to vary between 1 and 700 nM [13] depending on the cellular type and the specific system; so intracellular steady-state concentrations of H_2O_2 above 1 μM are considered to cause oxidative stress, inducing growth arrest and cell death [14,15].

The ROS exert a cytotoxic effects on somatic cells, increase the permeability of the blood-brain barrier and induce the peroxidation of lipids and cell membrane destabilization [16] causing altered function of critical enzymes involved in ion homeostasis, intermediary metabolism, cell repair and cellular death [17]. Additionally, these peroxidised lipids have been reported to accumulate in oxidative stressed individuals, playing an important role in ageing, as well as pathological processes such as diabetes and atherosclerosis and it is often the cause of free radical–mediated damage in cells in a feedback cycle with ROS [1].

Another well-established oxidative stress biomarker is the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFkβ), which translocates into the nucleus of the cells responding to inflammatory processes [18] and adaptive immunity conditions [19], and then binds to specific DNA sequences in the regulatory regions of its target genes, inducing the expression of a multitude of genes involved in inflammation and proliferation [20].

Under normal physiological conditions, a balance between ROS production and the antioxidant enzyme system is maintained. In order to keep a normal intracellular redox homeostasis several mechanisms

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have emerged, during evolution, to limit and neutralize excessive ROS and prevent it from damaging the cellular structure [1]. In large part this protection relies on the antioxidant substances, such as pyruvate or glutathione and in the activation of the Nrf2-Antioxidant Response Element (ARE) signalling pathway, which controls the expression of genes whose protein products are involved in the detoxification and elimination of reactive oxidants and electrophilic agents through conjugative reactions and by enhancing cellular antioxidant capacity [21,22]. Within this ARE group there are classical antioxidant enzymes such as: superoxide dismutases (SODs), which can directly generate and eliminate the hydrogen peroxide radical [23], catalases (CATs), and glutathione peroxidases (GPXs) [24]. Other important antioxidant enzymes of this system are hemeoxygenase (HMOX) [1], sulfiredoxins (SRXNs) [25], and peroxiredoxins, (PRDXs) [26,27]. The expression of antioxidant enzymes is also regulated by factors like Protein Kinase D1 serine/threonine Kinases (PRDK1), Kelch-Like ECH-Associated Protein 1 (KEAP1) and NFkβ signalling [28-30], being the latter activated by upstream molecules such as mammalian Target of Rapamycin (mTOR) in mammals [31]. Finally, it is important to consider metabolism regulating enzymes such Glutathione Synthetase (GSS) and senescence mediators like Tumour Suppressor Protein p53 (TP53), sensitive to oxidative conditions, playing a pivotal function in cellular apoptosis triggered by oxidative stress [32].

In this study we have used hESCs because for decades *in vitro* culture systems have been exploited to elucidate the mechanisms involved in acute oxidative stress and to analyse the protective effect of antioxidants, providing a huge amount of information [33,34]. However, very few data can be found in the literature explaining how long-term oxidative stress can affect the different cell types and most of our knowledge in this field is derived from differentiated cells, while hESCs have not been investigated. ESCs reflect the same features than ICM cells, showing for example similar mitochondrial morphology and mass, and meeting their energy requirements predominantly via anaerobic glycolysis [35,36]; so they constitute a good model to analyse the effect of oxidative stress in the early embryo. Examples of inappropriate environment inducing oxidative stress in the early embryo [37] are maternal diabetes, which now affects nearly 9% of population in the world [38-40], and Assisted Reproductive Technologies (ART), that allow the birth of about 5 million test tube babies [41] per year. The long-term impact of these suboptimal pre-implantation environments is a cause of concern that stems from the concept expressed in the Developmental Origins of Health and Disease theory (DOHaD) [42], which holds that inappropriate environment during the highly sensitive pre-implantation period, predispose to chronic diseases in adulthood by inducing epigenetic and gene regulatory networks changes [43].

Therefore, the objective of this study was to investigate the differential response between human somatic cells, fibroblast and endothelial cells, and ESCs against an *in vitro* oxidative stress treatment induced by H_2O_2 exposure in the non-cytotoxic range. To this aim we analyse ROS and lipid peroxidation levels, protein modifications and gene expression changes to demonstrate that somatic and ESCs show different responses, and to provide a novel model to study how the oxidative environment can affect the early embryonic cells.

Materials and Methods

Cell culture

Human fibroblasts (Hs27 cell line, obtained from Biobanking of Veterinary Resources, IZSLER, Brescia, Italy) were cultured in Dulbecco´s Modified Eagle Medium (DMEM, high glucose, GlutaMAX TM supplement, Gibco Invitrogen, Milan, Italy), supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich, St. Louis, MO, USA). Human Umbilical Vein Endothelial Cells (HUVEC cell line, obtained from Biobanking of Veterinary Resources, IZSLER, Brescia, Italy) were cultured in Medium-200 supplemented with 2% Low Serum Growth Supplement (Gibco Invitrogen, Milan, Italy). Cells were passaged 1:4 by 0.05% trypsin/EDTA incubation at 37 °C for 5 min every 3 or 4 days.

Human embryonic stem cells (hESCs) (HUES3 and HUES7 cell lines, obtained from Harvard Stem Cells Institute) [44] were first cultured on a feeder layer of mouse embryonic fibroblasts (MEFs) inactivated by mitomycin C (Sigma–Aldrich, Milan, Italy) in KO-DMEM medium (Gibco Invitrogen, Milan, Italy) supplemented with 10% serum replacement (Gibco Invitrogen, Milan, Italy), 4.3 mg/ml bovine serum albumin (BSA) (Sigma–Aldrich, Milan, Italy), 2 mM glutamine (L-alanyl-L-glutamine, Sigma–Aldrich, Milan, Italy), 1% non-essential amino acids (Gibco Invitrogen, Milan, Italy), 0.055 mM beta-mercaptoethanol (Gibco Invitrogen, Milan, Italy), 50 units/ml penicillin, 50 mg/ml streptomycin, and 10 ng/ml bFGF (Peprotech, Milan, Italy). To perform the experiments, hESCs were adapted to grow in feeder-free conditions in mTeSRTM1 medium (Stemcell Technologies, obtained from Voden medical instruments, Milan, Italy). The medium was changed daily and cells were passaged 1:4 with PBS/EDTA every 3 or 4 days.

The exposure to H_2O_2 started 24 h after plating and medium was changed daily during the following 72 h with concentrations tested ranging from 2 to 768 µM, ending at day 4 after plating. For cytotoxicity analysis, ROS and lipid peroxidation detection all cell lines were grown in 96-well plates. For gene expression analysis Hs27 and HUVEC cells were grown in 60 mm dishes and HUES cells in 24-well plates. To reach the optimal cell confluence after 72 h treatment, the cells were plated at different concentrations for each experiment: for general viability assay and gene expression analysis somatic cells (Hs27 and HUVEC) were plated at 60.000 cells/ml and hESC (HUES3 and HUES7) at 80.000 cells/ml. For ROS detection Hs27, HUVEC and HUES7 cells were plated at 10.000 cells/ml and HUES3 at 20.000 cells/ml, and for lipid peroxidation analysis all cell lines were plated at 20.000 cells/ml. For immunofluorescence detection cells were seeded on 6 mm diameter glass cover slides at a concentration of 80.000 cells/ml for all cell lines, and were treated 24 h post-plating with increasing concentrations of H_2O_2 during a period of 2 h to time enough to observe the direct NFkβ activation by H_2O_2 [19].

Cellular toxicity assessment by alamarBlue

Cellular toxicity was measured at the end of the 72 h treatment with a test based on the reduction of the alamarBlue® reagent as previously described [45]. Briefly, 10% of alamarBlue® (Molecular Probe, Invitrogen, Milan, Italy) diluted in culture medium was added to the cells and plates were incubated at 37°C in 5% of CO_2 for 6 h. Then, the absorbance was measured at 570 nm, using 600 nm as a reference wavelength, in a Tecan Infinite F200 PRO microplate reader (Tecan Italia srl, Cernusco sul Naviglio, Italy). Three different wells were analysed for each concentration of H_2O_2 treatment and three replicates were performed for each experiment.

ROS detection by CM-DCFDA probe

Intracellular ROS levels were measured by the 2',7'-dichlorodihydrofluorescein diacetate fluorometric assay (CM-DCFDA, Invitrogen, Paisley, UK). Briefly, this compound is typically loaded into cells in the form of a membrane-permeant diacetate (DA) ester, which is converted into a membrane impermeant product

inside the cell. Intracellular intermediate CM-H2DCFDA taken up by the cells is converted into non-fluorescent CM-H2DCF by esterase action and subsequently oxidized by intracellular oxidants into highly fluorescent CM-DCF. The levels of CM-DCF-forming ROS can be reliably determined by measuring the rate of CM-DCF formation [46]. By quantifying fluorescence, a fair estimation of the overall oxygen species generated under the different conditions can be obtained. After 72 h of treatment and removal of the medium the cells with an approximately 75% of confluence were washed once with TCM199 medium (Sigma-Aldrich, Milan, Italy) and incubated with 10 μM CM-DCFDA in TCM199 medium in the dark at 37°C in 5% CO_2 for 30 min. Then, cells were washed for three times with phosphate buffered saline (PBS), and fluorescence was measured in a Tecan Infinite F200 PRO microplate reader (Tecan Italia srl, Cernusco sul Naviglio, Italy) at 485 nm excitation and 535 nm emission wavelengths. Three different wells were analysed for each concentration of H_2O_2 treatment and three replicates were performed for each experiment.

Lipid Peroxidation analyses by BODIPY581/591 assay

Intracellular lipid peroxidation levels were measured by BODIPY581/591 C11 Assay (Image-iT Lipid Peroxidation Kit, Invitrogen, Paisley, UK). Briefly, this assay is dependent upon the sensitivity of the fluorophore BODIPY (581/591) C11 to oxidation by radicals (peroxyl and alkoxyl) formed from lipid hydroperoxides. The probe readily incorporates into biological membranes and responds to free radical attack with a spectral emission shift from red to green (from ~ 590 nm to ~ 510 nm), which can be readily monitored and quantified [47]. After 72 h of H_2O_2 treatment and removal of the medium, cells with an approximately 85% of confluence, were washed once with TCM199 medium without phenol red (Sigma-Aldrich, Milan, Italy) and incubated with 10 μM of lipid peroxidation sensor in TCM199 medium in the dark at 37°C in 5% CO_2 for 30 min. Then, cells were washed for three times with PBS supplemented with Calcium and Magnesium. After that, fluorescence was measured in a Tecan Infinite F200 PRO microplate reader (Tecan Italia srl, Cernusco sul Naviglio, Italy) at 485 nm excitation and 535 nm emission wavelengths. Three different wells were analysed for each concentration of H_2O_2 treatment and three replicates were performed for each experiment. Cells were observed by fluorescence microscopy (Nikon Eclipse 80i) and pictures are provided as "Supplementary Figure S1".

Fluorescence immunocytochemistry

In order to localize NFkβ transcription factor, cells were grown on glass cover slides, washed once with PBS and fixed in 4% paraformaldehyde (VWR, Milan, Italy) for 30 min at room temperature (RT). Then, they were permeabilized by incubation in 0.5% Triton (Sigma, Milan, Italy) in PBS for 15 min at RT and blocked in 10% normal goat serum (Sigma, Milan, Italy) in PBS for 1 h at RT. After that, cells were incubated overnight at 4°C in 1:100 rabbit anti-NFKβ antibody (Abcam, Cambridge, UK) and 1:1000 mouse anti-tubulin antibody (Sigma, Milan, Italy) to localize the cytoplasm. Following incubation, cells were washed three times and incubated in PBS containing 1:100 FITC anti-rabbit (Jackson ImmuneResearch, Milan, Italy) and 1:150 Texas Red anti-mouse antibodies (Jackson ImmuneResearch, Milan, Italy) for 1 h in the dark at RT. Finally, cells were incubated with 5 μg/ml Hoechst 33342 (Sigma, Milan, Italy) for 15 min in the dark at room temperature and washed three times in PBS and mounted with Citifluor (Citifluor Ltd., London, UK). Slides were observed by fluorescence microscopy (Nikon Eclipse 80i). Assessment of protein translocation to the nucleus was based on the comparison of the nuclear and cytoplasmic fluorescence intensity of the treated samples with the controls. Negative controls were performed with omission of the primary antibody before secondary antibody addition.

RNA Isolation, cDNA Synthesis and qPCR

RNA was extracted from the cells treated with the different H_2O_2 concentrations, from three different biological replicates for the somatic cells (Hs27 and HUVEC) and four for the hESC (HUES3 and HUES7), using the RNeasy Mini Kit (Qiagen, Milan, Italy) following the manufacturer's instructions. Immediately after extraction, the reverse transcription reaction was carried out with iScriptTM cDNA Synthesis Kit (Bio-Rad, Milan, Italy) following the manufacturer's instructions. Tubes were first incubated at 25°C for 5 min and then at 42°C for 30 min to allow the reverse transcription of RNA, followed by 85°C for 5 min to denature the enzyme. mRNA transcripts were quantified by real-time qRT-PCR. Three independent PCR replicates were conducted for all genes of interest. Experiments were designed to compare the relative levels of each transcript and those of the housekeeping gene 18S in each sample. PCR was performed with the PCR mix iTaqTM Universal SYBR Green Supermix (Bio-Rad, Milan, Italy) containing the specific primers (Supplementary Table T1) in a MyiQ Real-Time PCR Detection System (Bio-Rad, Milan, Italy). The comparative cycle threshold (CT) method was used to quantify expression levels. Quantification was normalized to the endogenous control 18S. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction wherein fluorescence increased above background for each sample. According to the comparative CT method, the ΔCT value was determined by subtracting the 18S CT value for each sample from the CT value of each gene in the sample. ΔΔCT was calculated using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to be subtracted from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula 2–ΔΔCT [48] and up or down regulation in gene expression was expressed relative to the control group.

Statistical analysis

All values are expressed as mean ± standard error media (SEM). Statistical analysis was performed on the data using the Student's t test to calculate significant differences between treated group samples compared with the control (CTR). $P \leq 0.05$ was considered statistically significant.

Results

Cytotoxic effects of oxidative stress induced by hydrogen peroxide in human somatic cells and hESCs

To evaluate the nominal concentration-effect relationship for the cytotoxic action of H_2O_2, human somatic cells (Hs27 and HUVEC) and embryonic stem (HUES3 and HUES7) cell lines were exposed to increasing concentrations of H_2O_2 between 4 and 768 μM during 72 h, and cell viability was analysed by alamarBlue® assay. Cytotoxic effect of H_2O_2 was similar in Hs27, HUES3 and HUES7 cells (between 16 and 32 μM), while HUVEC cells showed higher resistance with cytotoxic effect detectable at higher H_2O_2 concentrations (between 32 and 128 μM). The non-cytotoxic range (Figure 1A) was between 4 and 16 μM H_2O_2 for Hs27 and HUES cells and up to 32 μM for HUVEC cells (Figure 1A). At higher H_2O_2 concentrations, above 32 μM for Hs27 and HUES cells and gradually above 128 μM for HUVEC cells, the cell viability decreased drastically.

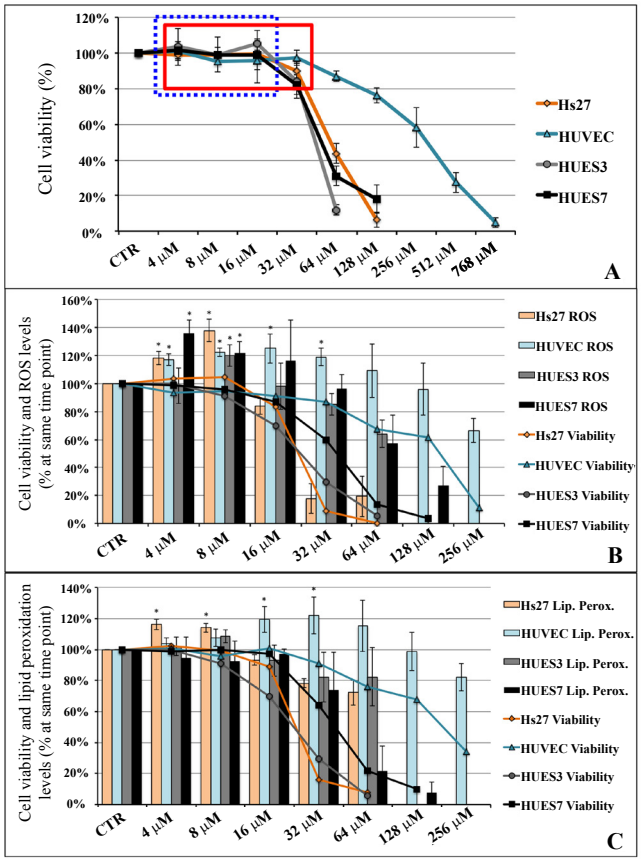

Figure 1: (A) Dose response curves following hydrogen peroxide (H_2O_2) exposure: Hs27 and HUVEC were plated at 60.000 cells/ml, HUES3 and HUES7 cells at 80.000 cells/ml and then were exposed to increasing concentrations of hydrogen peroxide for 72 h and cell viability was determined by alamarBlue® reagent. *Blue dotted square* highlights non-cytotoxic range for Hs27 and HUES cells; *solid red square* highlights non-cytotoxic range for HUVEC cells. Data (means ± SEM, 3 samples per H_2O_2 experimental condition, 3 separate replicates) are expressed as percentages of cell viability relative to the respective CTR, untreated control cells. **(B) Reactive oxygen species (ROS) generation by H_2O_2 treatment:** Hs27, HUVEC and HUES7 cells were plated at 10.000 cells/ml and HUES3 cells at 20.000 cells/ml and then were exposed to increasing concentrations of H_2O_2 for 72 h. Then intracellular ROS production was evaluated using CM-DCFDA, a membrane permeable non-fluorescent reagent that is converted into fluorescent DCF in the presence of ROS, and fluorescence was measured at 30 min. Cell viability was determined by the alamarBlue® reagent. Data (means ± SEM, 3 samples per H_2O_2 experimental condition, 3 separate replicates) are expressed as percentages of fluorescence increase relative to the respective CTR, untreated control cells. * Indicates statistically significant differences of non-treated cells compared to the treatment, two-tailed t-Test P ≤ 0.05. **(C) Lipid peroxidation generation by H_2O_2 treatment:** Hs27, HUVEC, HUES3 and HUES7 cells were plated at 20.000 cells/ml and then exposed to increasing concentrations of H_2O_2 for 72 h. Then intracellular lipid peroxidation levels were measured with BODIPY reagent a membrane permeable non-fluorescent reagent that is converted into fluorescent DCF in the presence of ROS, fluorescence from live cells shifts from red to green, providing a radiometric indication at 30 min. Cell viability was determined by the alamarBlue® reagent. Viability curves are slightly different between ROS and lipid peroxidation analysis depending on the plate cell concentrations. Data (means ± SEM, 3 samples per H_2O_2 experimental condition, 3 separate replicates) are expressed as percentages of fluorescence increase relative to the respective CTR, untreated control cells. * Indicates statistically significant differences of non-treated cells compared to the treatment, two-tailed t-Test P ≤ 0.05

Hydrogen peroxide treatment acts as powerful inducer of the intracellular increase of Reactive Oxygen Species levels

To investigate the effect of H_2O_2-induced oxidative stress, we measured the intracellular ROS generation using CM-DCFDA probe, a membrane permeable non-fluorescent reagent that is converted into fluorescent DCF in the presence of ROS. After 72 h of H_2O_2 treatment, cells were incubated with the CM-DCFDA reagent, and fluorescence was measured after 30 min (Figure 1B). In preliminary experiments accumulation of ROS was not detectable less than 2 μM H_2O_2 treatment. Then, a H_2O_2 dose-dependent ROS increase was observed for somatic and embryonic cell lines, reaching significance at 8 μM in HUES3 cells, between 4 and 8 μM in Hs27 and HUES7 cells and between 4 and 32 μM in HUVEC cells. Cell viability was analysed with the alamarBlue® test using the same cells concentrations used for the ROS assay. The increase of ROS levels occurred in the non-cytotoxic range for both somatic cells and hESCs, confirming that oxidative stress induces ROS accumulation and cell damage well before cell death becomes detectable.

Reactive oxygen species induce lipids peroxidation and their accumulation in somatic cells

To investigate the process of lipid peroxidation induced by ROS, we used Image-iT® Lipid Peroxidation Kit, based on BODIPY® 581/591 C11 reagent which is a sensitive fluorescent reporter for lipid peroxidation. Upon oxidation in live cells, fluorescence shifts from red to green, providing a ratiometric indication of lipid peroxidation by traditional microscopy systems. For quantification purposes, the entire population of cells displaying an increase in the green fluorescence was counted as positive (Figure 1C). The results revealed an influence of the H_2O_2 treatment in the lipid peroxidation levels, tendentially increased in the hESCs and significantly increased in Hs27 cells between 4 and 8 μM, and in HUVEC cells between 16 and 32 μM.

After measuring the fluorescent signal with a Tecan plate reader, a series of microscopy pictures of the somatic cells were taken demonstrating that the green signal, corresponding with the peroxidized lipids, increases with the H_2O_2 treatment in comparison with the CTR, control non-treated cells (Supplementary Figure S1). Cell viability was analysed in parallel with the alamarBlue® test using the same cell concentrations that for the lipid peroxidation assay. The increase of lipid peroxidation levels occurred in the non-cytotoxic concentrations of H_2O_2 and was more clearly detectable in the somatic cell lines than in the embryonic cells.

H_2O_2 induces ROS accumulation and the activation of NFkβ depending mechanisms against oxidative stress inside the cell

To study if H_2O_2 treatment modulates oxidative stress gene expression, we analysed the localization of the NFkβ protein, whose translocation from the cytoplasm to the nucleus is induced by oxidative stress, regulating in this way the expression of several genes that codify for antioxidant enzymes [19]. NFkβ factor translocation was observed both in somatic and embryonic cells, at the not cytotoxic H_2O_2 concentrations, being more evident between 32 and 64 μM H_2O_2 in a short treatment of two hours, where the cells keep unaltered their morphology and number comparing with the control (Figure 2). The translocation of NFkβ occurred in all cell types in the same way, being more visible in the two embryonic cell lines (Figures 2F and H). Complete panels of NFKB translocation pictures, with all the H_2O_2 concentrations treatment for Hs27 (Supplementary Figure S2), HUVEC (Supplementary Figure S3), HUES3 (Supplementary Figure S4) and HUES7 (Supplementary Figure S5).

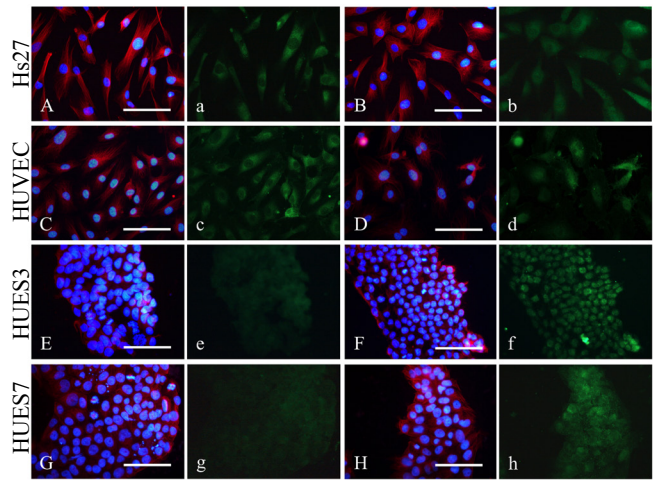

Figure 2: NFkβ translocation comparison between non-treated and H_2O_2 treated both somatic and embryonic stem cells. 24 h post plating cells were treated for 2 hours with increasing concentrations of H_2O_2, then were fix and immunofluorescence staining was performed with an anti-NFkβ (green) and an anti-tubulin (red) antibody and Hoechst 33342 (blue) was used for nuclei localization. To create this figure has been chosen representative pictures from non-treated and treated cells, selecting the H_2O_2 concentration in which was observed the maximum effect in terms of NFkβ translocation. Representative pictures from A to H panels show the merged images of anti-tubulin and Hoechst staining, and from a to h panels show anti- NFkβ staining. H_2O_2 conditions: **(A, a)** Hs27 non-treated cells, **(B, b)** Hs27 – 64 μM; **(C, c)** HUVEC non-treated cells, **(D, d)** HUVEC 64 μM; **(E, e)** HUES3 non-treated cells, **(F, f)** HUES3 – 64 μM; **(G, g)** HUES7 – non-treated cells, **(H, h)** HUES7 – 32 μM.
Scale bars=100 μm

High ROS levels induced by H_2O_2 modulate gene expression of ARE genes in somatic and embryonic stem cells at not cytotoxic concentrations

To unravel the enzymatic antioxidant mechanisms against the non-cytotoxic concentrations of H_2O_2, during 72 h exposure, the expression levels of a total of 18 genes (Supplementary Table T1) were analysed by qPCR in the two somatic and two embryonic stem cell lines. In particular Hs27 cells were analysed following exposure to hydrogen peroxide between 4 and 32 μM, HUVEC between 8 and 64 μM and HUES cells between 4 and 16 μM. All selected genes were investigated to examine their expression profile due to their function in processes related to oxidative stress, metabolism, inflammation and apoptosis pathways. Graphs in Figures 3 and 4 shows only the genes in which we observed significant changes in one or more of the concentrations tested for each cell line.

Oxidative stress and metabolism: GPX1 and CAT, two of the classical main antioxidant enzymes, were upregulated by H_2O_2 treatment in both somatic (Figures 3A, 3C, 3J and 3K) and embryonic (Figures 4A and 4C) cell types. Also the GPX3 isoform, mainly present in the extracellular space, was upregulated in H_2O_2 exposed HUVEC and embryonic cells compared with the control (Figures 3P and 4G). Similar response was observed with HMOX, which catalyses the degradation of heme groups, and which activity was also enhanced in HS27 and embryonic cells (Figures 3B and 4B).

Next, we examined SOD2 and 3 which catalyse the dismutation of superoxide radicals in less reactive species and have been described also as classical antioxidant enzymes [19]. The isoform SOD3 showed significant upregulation for both embryonic cells lines (Figure 4E), but no significant differences were observed in somatic cells.

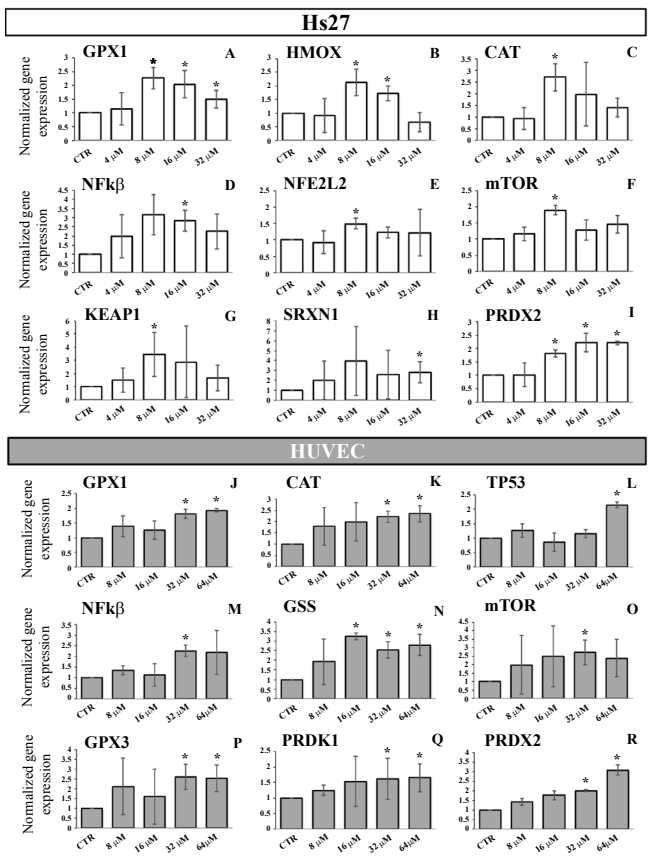

Figure 3: Gene expression in somatic cell treated with hydrogen peroxide (H_2O_2): ARE genes and cell damage-related genes. Relative gene expression between control and 72 h H_2O_2 treatment. A–I: Hs27 cells: CTR, untreated control cells, 4 μM, 8 μM, 16 μM and 32 μM. J–R: HUVEC cells: CTR, untreated control cells, 8 μM, 16 μM, 32 μM and 64 μM. Genes: **(A)** Glutathione peroxidase 1, **(B)** Hemeoxygenase, **(C)** Catalase, **(D)** Factor kappa-light-chain-enhancer of activated B cells, **(E)** Erythroid-derived 2-like 2, **(F)** Mammalian target of rapamycin, **(G)** Kelch-Like ECH-Associated Protein 1, **(H)** Sulfiredoxin 1, **(I)** Peroxiredoxin 2. **(J)** Glutathione peroxidase 1, **(K)** Catalase, **(L)** Tumour suppressor protein p53, **(M)** Factor kappa-light-chain-enhancer of activated B cells, **(O)** Glutathione Synthetase, **(P)** Glutathione peroxidase 3, **(Q)** Protein kinase D1 serine/threonine kinase, **(R)** Peroxiredoxin 2. Samples were normalized on the untreated control. Only genes having differential expression in one or more H_2O_2 concentrations tested are shown for each cell line.
*Indicates statistically significant differences of non-treated cells compared to each treatment, two tailed t-Test P ≤ 0.05

Another group of antioxidant enzymes are the peroxiredoxins, which contain essential catalytic cysteines that use thioredoxin to scavenge H_2O_2, and provide a potent defence mechanism that maintains the redox balance in normal and oxidative stress conditions [26]. In particular the isoform 2, PRDX2, was described upregulated in diabetic patients versus healthy control subjects [27], and this is the reason why we considered this gene of interest. The expression was higher in treated cells of all the four cells lines analysed, with a similar increase of gene expression activity across the range analysed for both somatic (Figures 3I and 3R) and embryonic cells (Figure 4 I).

We also analysed the expression of SRXN1 which belongs to a family of genes coding for oxidoreductase enzymes involved in antioxidant metabolism by re-activating peroxiredoxins [25]; we found that its activity was increased in Hs27 treated cells (Figure 3H).

Then we analysed PRDK1, that regulates a variety of cellular functions, including protecting mitochondria from oxidative stress

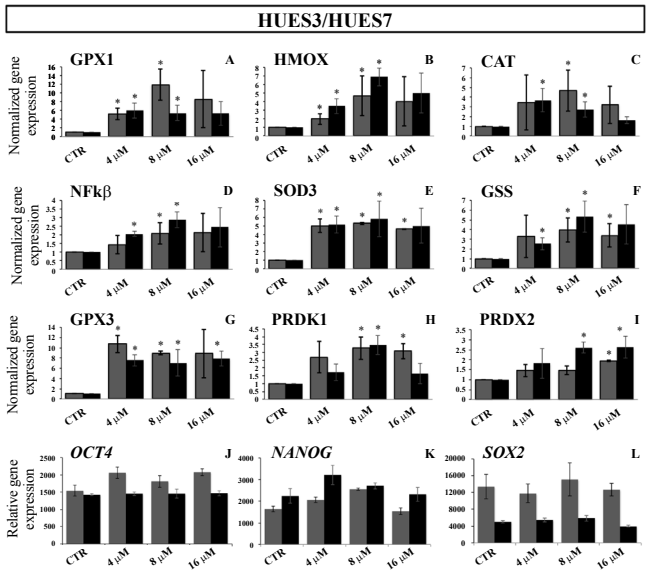

Figure 4: Gene expression in hESC treated with hydrogen peroxide (H_2O_2): ARE genes and cell damage-related genes. Dark grey columns represent HUES3 cells and black columns represent HUES7 cells. Relative gene expression between CTR, untreated control cells and 4 μM, 8 μM and 16 μM H_2O_2 conditions of 72 h treatment. Genes: **(A)** Glutathione peroxidase 1, **(B)** Hemeoxygenase, **(C)** Catalase, **(D)** Factor kappa-light-chain-enhancer of activated B cells, **(E)** Superoxide dismutase, **(F)** Glutathione Synthetase, **(G)** Glutathione peroxidase 3, **(H)** Protein kinase D1 serine/threonine kinase, **(I)** Peroxiredoxin 2. Samples were normalized on the untreated hESCs control. **(J–L)**: Gene expressions of pluripotency genes (OCT4, NANOG and SOX2). Only genes having differential expression in one or more H_2O_2 concentrations tested are shown for each cell line.
*Indicates statistically significant differences of non-treated cells compared to each treatment, two tailed t-Test P ≤ 0.05

[29], and we found a significantly higher expression in HUVEC and in embryonic treated cells (Figures 3Q and 4H).

Finally we examined NFE2L2 (encoding nrf2 transcription factor) and KEAP1. The former plays a main role in the day-to-day biological response to oxidative stress, regulating the transcription of many antioxidant genes that preserve cellular homeostasis and also of detoxification genes that process and eliminate toxins before they can cause damage. The latter, KEAP1, is defined as an interacting partner of NFE2L2 gene, because under oxidative stress conditions abolishes the inhibition of Nrf2 that become stabilized, translocates and accumulates in the nucleus, where it binds to the ARE in the enhancers of its target genes, leading to a general cytoprotective response. In our study we found that both NFE2L2 and KEAP1 genes were upregulated in Hs27 cells exposed to hydrogen peroxide (Figures 3E and 3G).

Significance differences were observed as well in the expression of GSS gene, directly responsible of glutathione metabolism, in treated HUVEC and HUES cells (Figures 3N and 4F).

Inflammation: We then analysed the main player of the inflammation pathway mTOR, demonstrating higher expression levels in treated somatic cells (Figures 3F and 3O); its activity has been described related with the NFkβ gene which level was also significantly higher in somatic (Figure 3D and 3M) and in embryonic cells exposed to H_2O_2 (Figure 4D). These interesting effects of our 72 h oxidative stress treatment are in agreement with the description of H_2O_2 as a direct inducer of the transcription factor NFkβ, a key regulator of the inflammatory process and adaptative immunity.

Apoptosis and cell death: To investigate the role of apoptosis in our oxidative stress model we analysed TP53, a gene that encodes a protein regulating cell death induced by DNA damage and we found that its expression was significantly increased in HUVEC treated cells (Figure 3L).

Finally, we checked the expression level of the pluripotency genes OCT4, NANOG and SOX2 in hESCs within the not cytotoxic range. No differences were found between the H_2O_2 treated (Figures 4J-4L) and the untreated cells, demonstrating that the oxidative treatment applied in this study was not affecting the main regulatory network of pluripotency.

In summary, our model demonstrates that H_2O_2 treatment induces the modification of crucial oxidative stress biomarkers like ROS and lipid peroxidation levels, and mobilizes several antioxidant enzymes through NFkβ translocation in all the cell lines observed. Moreover this *in vitro* model highlights differences between somatic and embryonic cells in their antioxidant response towards H_2O_2 induced damage.

Discussion

Oxidative stress: Viability, ROS and lipid peroxidation effects

It has been described that oxidative stress causes a very wide spectrum of genetic, metabolic and cellular responses, being necrosis, which is the most extreme outcome, the only one that involves direct cell destruction [49]. Most oxidative stress conditions that cells might actually encounter have non-visible morphological effects and modulate changes in membrane lipid peroxidation, gene expression and induce several transient adaptive responses. This study provides the evidence that 72 h exposure to H_2O_2 generates an oxidative stress state, which causes different effects in human somatic cells versus embryonic stem cells. Compartmentalization of function might influence how cells respond to stress: in our study, HUVEC were the most resistant cells in terms of viability and therefore accumulated ROS and peroxided lipids at higher concentrations of H_2O_2. This could be linked to the fact that endothelial cells *in vivo* are continuously exposed to shear stress, which has an important impact on cellular structure, function and metabolism [50], ultimately making them stronger against damage caused by H_2O_2 and potentially more active in its elimination.

An important fact to take into account in commenting the results of the present study is cell density, because cytotoxicity is inversely proportional to cell confluence [51]. Here ESCs were plated at higher concentrations than somatic cells because preliminary experiments determined the optimal concentrations for each cell type to reach cell confluence in our experimental design. However, ESCs did not show more resistance to H_2O_2 exposure. In relation to the medium used for the culture, we selected for hESCs and HUVEC specifically designed serum-free media formulations while for fibroblasts we used a serum-supplemented medium. These different culture protocols for the different cell lines are widely used in the literature and recommended to achieve the best culture conditions for each cell type [52,53].

One of the strengths of our model is the design of a strict experimental protocol for the use of H_2O_2, in order to generate reproducible results avoiding inconsistent effects due to its well-known instability. The relevance of H_2O_2 resides in the fact that depending on concentration and time of exposure different effects are elicited. With lower exposure the generation of ROS affects the cell cycle and induce the entrance into G0. With long exposure and consequent accumulation of high levels of ROS the apoptosis mechanisms are triggered mediated by increased expression of genes such TP53. In cycling cells, p53 dephosphorylates

in response to oxidants resulting in cells arrested in S-phase [54]. Interestingly, HUVEC was the only cell line in which we have seen high expression of TP53 in treated cells. This high expression could be explained by the gradual slope of the viability curve in comparison with the other somatic and embryonic cells lines.

In our study, we confirm that external H_2O_2 treatment generates an oxidative stress environment, which produces an extra accumulation of ROS in both somatic and embryonic cells. It is now apparent that a very complex intra-cellular regulatory system involving ROS exists within cells and that these agents play an important role in ageing, chronic diseases and cancer [55]. With low and medium doses of H_2O_2, at sub-lethal oxidative concentrations, we found an accumulation of ROS and lipid peroxidation at higher H_2O_2 concentrations in HUVEC cells than in Hs27 and HUES lines, due to the higher resistance of somatic cells against H_2O_2. In these non-cytotoxic ranges resides the interest of our model because no changes of cell morphology are seen, cell proliferation is not affected and expression of plurypotency genes in hES cells is maintained. Therefore, it can be inferred that our model mimics the exposure of the early embryo to an oxidative environment *in vivo* such as during gestational diabetes or obesity, conditions in which anyway embryonic development takes place apparently normally and offspring is born.

The reason to assess the lipid peroxidation process, one of the most widely used indicators of free radical formation and a good biomarker of oxidative stress, is because it is a key factor in the generation of redox imbalance, favouring the formation of several toxic products such as malondialdehyde and 4-hydroxynonenal, which can attack lipids, but also DNA and proteins, predisposing to increased cardiovascular risk, carcinogenicity and mutagenicity [56]. In this study, in agreement to the literature, lipid peroxidation levels increased in all cells but beyond significance only in Hs27 and HUVEC somatic cells. This could mean that the embryonic cells have special, or at least different, damage removal systems in order to avoid that peroxidation and accumulation of unsaturated lipids affect cell membrane properties and signal transduction pathways, potentially inducing long-term effects and predisposition to chronic disease risk in later life. Besides, this difference might be linked to the lower basal intracellular levels of ROS in hESC as compared to fibroblast and endothelial cells [57,58]. Therefore, the same H_2O_2 treatment generates a direct increase of ROS in all cell types, but in hESC this accumulation does not induce significant lipid peroxidation. According to other authors [59,60] this condition could lead to higher resistance of hESCs to oxidative stress upon exposure to H_2O_2 compared to their differentiated somatic progeny.

Oxidative stress involves different gene expression and metabolic routes

Alterations in the cellular redox status modify DNA by transactivation activities of a variety of transcriptional mediators such as high ROS levels or peroxidised lipids. Given the oxidative stress status, the challenge for the cell is to develop antioxidant defences in order to survive. This, in turn, is driven by changes in expression of a variety of target genes with downstream effects on cell function and cytological features like telomere length and its influence on cellular life span [15,61]. Redox regulation of gene expression therefore appears to be a robust regulatory system that allows cells to adapt to environmental changes.

In this study we have shown that somatic cells and embryonic stem cells respond to H_2O_2 exposure through different transcriptomic and proteomic mechanisms. One of these main molecular routes is the NFkβ pathway that is activated in oxidative stress conditions and during the ageing process by high levels of cytoplasmic H_2O_2 and other ROS, resulting in a pro-inflammatory shift in gene expression profile [62]. In our model, NFE2L2 gene expression levels were significantly increased in all four lines and the nuclear translocation of NFkβ protein was observed in both somatic and embryonic lines, but more clearly in HUES cells [19]. Our findings in agreement with published data support the role of ROS as common activators of NFkβ, demonstrating that elevated levels of ROS are induced by peroxidised lipids that in turn are potent NFkβ-activating agents; moreover, antioxidants have been shown to block both ROS production and the resultant NFkβ activation [63]. Ongoing experiments by microarray and protein analysis are investigating other downstream-activated genes and pro-inflammatory enzymes actively involved in the formation of ROS.

It has been demonstrated that in disease conditions H_2O_2 plays a role in the activation of inflammatory gene pathways such mTOR, through the translocation of NFkβ to the nucleus [64]. Interestingly, the gene expression levels of mTOR was significantly increased in somatic cells, but not in embryonic cells, confirming that the antioxidant response takes place in all cell types, but following different mechanisms and triggering diverse effects.

NFkβ is also cross-talking with the nrf2 pathway. Activation of nrf2 pathway protects the cells and contributes to proliferation and survival of damaged cells, whereas its inhibition results in increased ROS production and cell damage. These pathways interface at several points to modulate the transcription of downstream targets in relation to the level of ROS and genes having AREs responsive to nrf2 often have also NFkβ binding sites [28]. An example is HMOX1, one of the most relevant phase II antioxidant enzymes. Our results showed that exposure to H_2O_2 induce the elevation of mRNA expression of this antioxidant gene in Hs27 somatic cells and embryonic cell lines, indicating the activation of the nrf2 pathway. Other authors have demonstrated an increased response of HMOX against oxidative stress in several somatic cell lines [65,66]. Interestingly in HUVEC cells this upregulation was not observed but this finding can be justified according with other reports on experimental diabetic animals, in which no major variation of the HMOX activity was observed versus healthy controls [67].

Nrf2 it is also directly involved in the upregulation of many other genes through the ARE sequences such as sulfiredoxins, peroxiredoxins and gluthathione related enzyme [68]. SRXN1 in this study resulted upregulated in Hs27 somatic cells. Furthermore this somatic cell line shows an increased expression of both KEAP1 and NFE2L2, fact that was also described in animals with high carcinogenic and cardiovascular risk [69], but the reason of this upregulation is not clear. Instead in the other cell lines SRXN1 was not increased, but it was noted the upregulation of GSS, indicating that different cells lines could activate different mechanism of defence. To address the question of why the expression of glutathione synthetase was different between cell lines, showing significant higher levels only in endothelial and embryonic cells, it is important to take into account the differences in metabolism and energy production. It has been reported that cells having high proliferation rate, like embryonic stem cells, require a defined metabolic pathway justified by the Warburg effect, in which cells meet their energy requirements through anaerobic glycolysis followed by lactate fermentation to produce ATP instead of aerobic oxidation in the mitochondria [70]. Instead our results showed elevated levels of peroxiredoxin 2 in all treated cell lines analysed, suggesting a possible highly conserved adaptive response to inflammatory stimuli. Inflammation often complicates diseases associated with oxidative

stress like diabetes mellitus, so this enzyme acts as redox-dependent inflammatory mediator both *in vivo* and *in vitro*, modifying the redox status of cell surface receptors and enabling induction of inflammatory responses [26]. So, our findings of significant increased expression in both somatic and embryonic cells support the DOHaD concept and the existence of an interesting crosstalk between PRDX isoform 2 and long-term metabolic diseases and cardiovascular risks [27]. Moreover this antioxidant protection against oxidative stress, also suggest a novel therapeutic mechanism for treating metabolic disorders [71].

Catalase and glutathione peroxidase enzymes constitute the main response in acute hyperglycaemia contributing to the maintenance of a normal intracellular redox homeostasis [1]. The biological importance of the increase of these two antioxidant enzymes resides in the fact that catalase, localized in intracellular peroxisomes and in the cytosol [72], reduces H_2O_2 to H_2O and O_2 through a two-step reaction; so it is thought to be important in severe oxidative stress by reducing intracellular H_2O_2, which is the by-product of $O_2^{\bullet-}$ self-interaction [73]. Glutathione peroxidase also reduces H_2O_2 and together with lipid peroxidases convert it to H_2O and lipid alcohols [72]. Its inadequate expression or dysfunction can contribute to OH- formation by not detoxifying H_2O_2 [74]. In our study, we confirmed the modification of both enzymes, being upregulated in all cell lines by the H_2O_2 treatment.

Within this group of classical antioxidant enzymes there is the superoxide dismutases family that catalyse the dismutation of H_2O_2 into O_2 and their activity is cell and tissue dependent [75]. In our study, human ESCs showed high levels of SOD3 gene expression in H_2O_2 treated cells compared with the control, but not changes of SOD1 and SOD2 expression (data non shown). Future studies will be needed to investigate the mechanism by which hESCs activate this ROS protective enzyme more efficiently or at least in a different way, than somatic cells.

Finally, accordingly to previous results [76], oxidative stress, within the not cytotoxic range, did not affect the expression of pluripotency genes, demonstrating that the oxidative treatment, in the not cytotoxic range, was not affecting the pluripotency of the embryonic cell lines and therefore, most likely, although not tested in this study, their ability to differentiate into cells and tissues of the three primary germ layers.

Conclusion and Relevance of this Model

The implications of an exposure to oxidative stress during embryonic development that is a common situation in assisted reproductive technologies (ART) or in pregnant diabetic mothers, is still largely unknown in humans and remains to be elucidated. Human ESCs are a unique biological tool to study and model *in vitro* how environmental alterations can affect the pluripotent cells of the early embryo in the critical developmental window between the early cleavage stages and the blastocyst stage inducing damage that can predispose to adult diseases according to the Developmental Origins of Health and Disease (DOHaD) theory [42].

Therefore, this study for the first time describes a model of non-cytotoxic oxidative stress conditions, during a period of 72 h, in human embryonic stem cells. This model demonstrates increased ROS levels, lipid peroxidation and differential modulation of gene expression and metabolic pathways between hES and somatic cells, providing novel insights for the understanding of the peculiar responses of human embryonic cells. Finally the findings presented here can contribute to optimise preventive antioxidant strategies to protect the cells of the early embryo exposed to environmental stress.

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Disclosure Policy

The authors declare that there is no conflict of interest regarding the publication of this paper.

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