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Mimicking of glutathione peroxidase deficiency by exposition of JAR cells to increased level of synthetic hydroperoxide

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A short chain synthetic analogue of lipid hydroperoxides was used to overload glutathione peroxidase (GPx) in human choriocarcinoma *cell line JAR cells. Cells exposed to 100 μM* tBuOOH *displayed a 40% reduction in ATP level and significantly increased in membrane permeability, visualised by the lactate dehydrogenase (LDH) release into the extracellular medium. The intracellular level of oxygen free radicals measured as an oxidation of the dichlorodihydro-fluorescein diacetate (H_2DCF-DA) significantly increased after 2 hours of cell exposition to 100 μM* tBuOOH*. Concomitantly MDA, 4-HNE level increased to 2 nmol/mg of cell protein after 2 hours. Mitochondria stained with MitoTracker Red CMXRos displayed a filamentous appearance in control cells but changed into granular less energised organelles after exposition to* tBuOOH*. Collectively, the above results indicate the importance of the contribution of oxidative stress in the development of pre-eclampsia.*

Key words: mitochondria, *choriocarcinoma*, oxidative stress, pre-eclampsia

INTRODUCTION

Lipid hydroperoxides (LOOH) are ubiquitous components of biological membrane. Placental trophoblast cells are recognised as the main source of endogenous LOOH [16]. A deficiency of placental glutathione peroxidase (GPx) activity can possibly increase the level of tissue peroxides being stimulatory to the formation of placental thromboxane [6, 23] and LOOH [25] and ultimately contributing to the development of pre-eclampsia. Hydroperoxides appear to play a significant role in lipid peroxidation, being both a product of the initial phase of lipid peroxidation as well as a main substrate for its further propagation. The aim of the present study was to inspect the effect of a short chain synthetic peroxide, namely *tert*-butylhydroperoxide (*t*BuOOH), on the energetic state of *choriocarcinoma* cells, the generation of free radicals inside the cell and the morphology of mitochondria as investigated by confocal microscopy.

MATERIAL AND METHODS

Cell line

Human *choriocarcinoma* cells JAR (ATCC HTB-144) were routinely cultured in 75-cm^2 plastic flasks (Sarsted) in RMPI 1640 containing 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 4.5 g/L glucose, supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 100 IU/ml penicillin and

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100 μg/ml streptomycin. The cells were grown in a humidified atmosphere with 5% CO_2 at 37°C. They were then passaged weekly by trypsin 0.25% and EDTA 0.03%.

Reagents

RMPI 1640 and *t*BuOOH were purchased from Sigma Aldrich Chemical (USA). The foetal bovine serum was produced by Gibco BRL (Grand Island, NY, USA). Trypsin with EDTA and phosphate buffer saline (PBS) pH 7.4 were products of Sigma Chemicals (USA). Mito Tracker Red CMXRos (CMXRos) Molecular Probes, Inc. FITC — Phalloidin was produced by Sigma P5282 (USA). Formaldehyde and glutaraldehyde were purchased from Merck (Germany), Perma Fluor mounting solution from Immunon (Pittsburgh, PA, USA) and Triton X-100 from Sigma Aldrich Chemical (USA).

Treatment of cells with tert-butylhydroperoxide

Cells were plated in 6-well plates at a concentration of 5×10^5–1×10^6 cells/well (0.5–1 mg protein/ /ml/well). The cells were exposed to 100 μM *t*BuOOH at specific times (control = 0 h, 1 h, 2 h).

Viability assay

Cell viability was evaluated by lactate dehydrogenase (LDH) release. After substracting the relevant volume of medium from above the cells (to be used as reference in the following steps), the cells were lysed by the addition of Triton X-100 (final concentration in culture dish 1%). The aliquots of the lysed cells as well as the reference culture medium were centrifuged at 1500 × g for 3 min and supernatants were used for the assay. The assay solution was added to a cuvette containing 0.05 M Tris-HCl buffer, pH 7.4, 1 mM sodium pyruvate and 0.15 mM NADH, and spectrophotometrical analysis was performed at 340 nm. LDH release was expressed as a percentage of total release, the release, that is, of LDH after cell lysis by 10% Triton X-100 [9, 13].

Measurement of intracellular ATP level

Sample preparation: Cells (~2×10^6 cells/ml) were collected by trypsinisation, centrifuged together with those floating in the culture medium and washed with PBS. The cells were then suspended in cold 1.4 M $HClO_4$ and incubated for 15 min on ice to extract cellular nucleotides. Acid extracts were centrifuged (14,000 RCF — 5 min), transferred to new tubes and neutralised to pH 6.5 by the addition of cold 3 M K_3PO_4 prior to centrifugation (14,000 RCF — 5 min). The supernatants were subjected to further analysis by HPLC. The method employed for HPLC determination of the intracellular level of ATP was based on that described by Smoleński et al. [21]. Protein concentration was evaluated using the Bradford method [4] after dissolving the perchloric acid precipitates with 0.5 M NaOH.

Staining and Visualisation of Mitochondria by Confocal Microscopy

Cells growing on 24 × 24 mm-glass coverslips under various experimental conditions were incubated with 100 nM Mito Tracker Red CMXRos (CMXRos; Molecular Probes, Inc.) for 30 minutes at 37°C in an atmosphere of 5% CO_2. The cells were fixed with a fixative containing 2% glutaraldehyde and 2% formaldehyde in PBS for 30 minutes at room temperature and then washed in PBS and permeabilised with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. This was followed by 4 washes in PBS. The coverslips with the cells were placed on glass slides in a Perma Fluor mounting solution (Immunon, Pittburg, PA) and analysed by a confocal system Radiance 2100 (Bio-Rad, UK), equipped with Krypton/Argon lasers and mounted on an Eclipse 600 (Nikon, Japan) microscope, using the software LaserSharp 2000 version 4.0 (Bio-Rad, UK) as described previously [11].

Flow cytometric determination of Reactive Oxygen Species and light scatter

Levels of Reactive Oxygen Species (ROS) were measured by flow cytometry as the fluorescence of DCF, which is the oxidation product of H_2DCF-DA with a sensitivity to ROS (H_2O_2, ROOH, NO). Cells were incubated for 30 min with the probe (20 μM) at 37°C. Cell samples were then fixed (1% paraformaldehyde), cooled (4°C), and protected from light for later analysis (cold-fixed cell samples). A FACSCAN (Coulter Elite) flow cytometer was applied to measure the ROS levels in the cells. Signals were obtained using a 530-nm bandpass filter (FL-1 channel) for DCF. Each determination was based on a mean fluorescence intensity of 7,000 cells [1, 20].

Measurement of lipid peroxidation products

Cells (~10×10^6 cells/ml) were collected by trypsinisation, centrifuged and washed with PBS. They were then suspended in PBS and the concentration of lipid peroxidation products measured. Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured using a lipid peroxidation

assay kit (Calbiochem, San Diego, CA) [7]. Protein concentration was evaluated [4] and the lipid peroxidation products were described as MDA, 4-HNE/mg protein.

RESULTS

Effect of *t*BuOOH on intracellular levels of ATP and LDH release

Time-dependent changes of intracellular levels of the ATP in *t*BuOOH-treated cells are shown in Figure 1. Two hours after the treatment, ATP levels had decreased to about 60% of the control value (control ≈ 12 nmol/mg protein) (Fig. 1). We noticed an increase in the permeability of the cell membrane to LDH, which was already distinct 2 hours after cell treatment (Fig. 1) and increased continuously in an extracellular medium thereafter to approximately 40% after 6 hours (result not shown).

Effect of *t*BuOOH on morphological changes of the mitochondria in choriocarcinoma cells

The mitochondria in the control cells stained with fluorescent dye CMXRos showed thin filamentaous structures (Fig. 2A) under a confocal laser microscope. JAR cells exposed to 100 μM *t*BuOOH for 2 hours (Fig. 2B) showed a mixed population of granular mitochondria of different sizes, in many cases distinctly enlarged. Exposition of cells to *t*BuOOH also resulted in notable changes in the shape of the cells as well as in perturbation of the plasma membrane integrity (Fig. 2B).

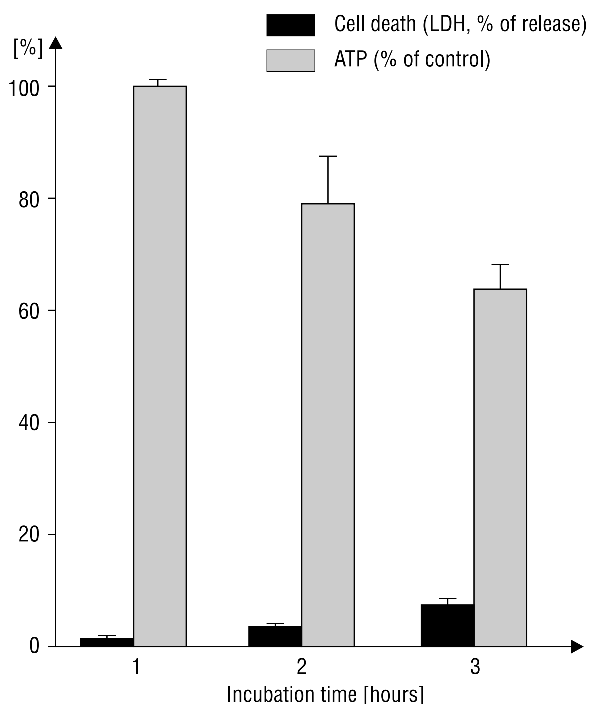
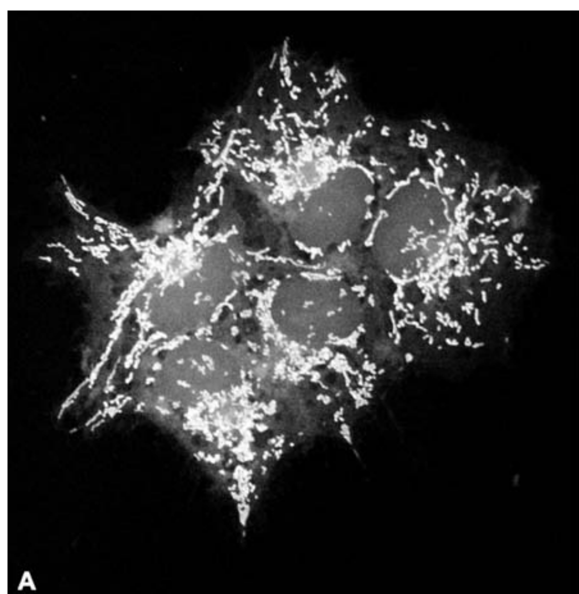


Figure 1. LDH release and the concentration of ATP in JAR cells after treatment with 100 μM *t*BuOOH. The values represent means ± SEM from 3 independent experiments.



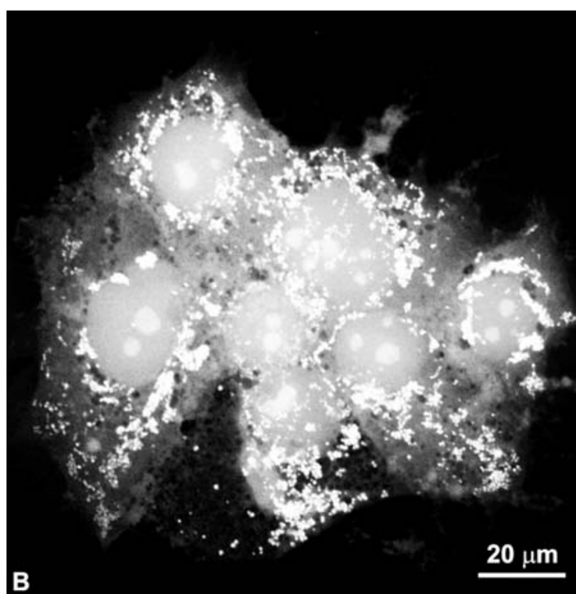


Figure 2. Morphological changes of mitochondria in JAR cells after treatment with 100 μM *t*BuOOH. **A.** Control cells; **B.** Treated with 100 μM *t*BuOOH for 2 hours.

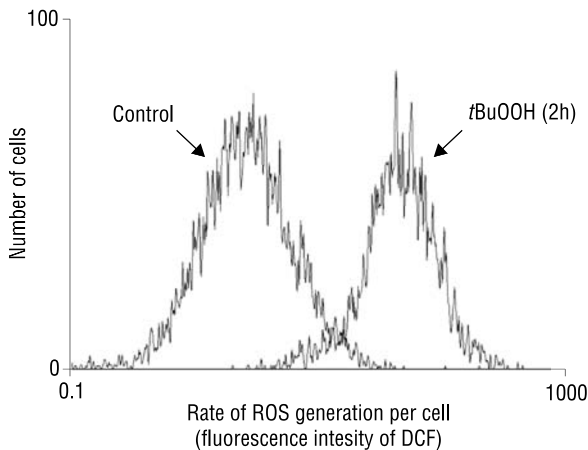


Figure 3. Changes in the intracellular levels of reactive oxygen species in JAR cells after treatment with 100 μM *t*BuOOH.

Effect of *t*BuOOH on the rate of generation of ROS from choriocarcinoma cells

A typical example of a flow cytometric histogram obtained from untreated control and *t*BuOOH-treated JAR cells is shown in Figure 3. A remarkable shift in the peak intensity of DCF to the right was already detected at 2 hours of *t*BuOOH treatment, indicating that intracellular levels of ROS were distinctly increased by *t*BuOOH treatment. As a consequence of the significant generation of ROS, an elevated level of MDA and 4-HNE (10-fold) was observed after 2 hours of cells exposition to *t*BuOOH (Fig. 4).

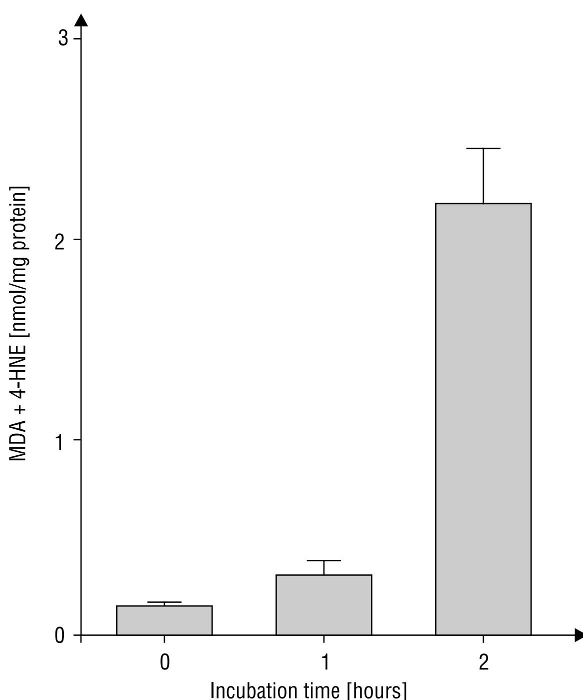


Figure 4. Changes in the intracellular levels of lipid peroxidation products (MDA, 4-HNE) in JAR cells after treatment with 100 μM *t*BuOOH. The values represent means ± SEM from 3 independent experiments.

DISCUSSION

We have shown in the present study that the *t*BuOOH-substance with well defined pro-oxidant properties induces mitochondrial stress in human *choriocarcinoma* cells. Generation of free radical species from *t*BuOOH was noticed after a 2-hour exposition of cells to the pro-oxidant. This indicates an imbalance in antioxidant defence, in which glutathione peroxidase plays a significant role, and the generation of ROS. Mitochondria are the main intracellular source of ATP and ROS [10, 15]. It has been demonstrated that mitochondria are mainly responsible for the biotransformation of *t*BuOOH into free radical products [12, 17]. As the result of an imbalance between the generation of ROS and antioxidant defence, cellular energetic catastrophe is created and the intracellular ATP level decreases abruptly, reaching 60% as early as 2 hours after the treatment. As a consequence of the depletion of ATP, *choriocarcinoma* cells start to lose the integrity of the plasma membrane and LDH starts to escape from the cell. In our experimental conditions, the increased level of the hydroperoxides definitively overwhelms the capacity of placenta glutathione peroxidase, the enzyme which is manly responsible for the "safe" two-electron reduction of hydroperoxides. Under these circumstances one-electron oxidation or a reduction in *t*BuOOH may create either the peroxyl *t*BuOO$^{\bullet}$ or alkoxyl *t*BuO$^{\bullet}$ radical respectively [5]. The mechanism for the reaction of cytochrome c with *t*BuOOH was investigated by Barr and Mason by ESR spin trapping using DMPO [3]. From these analyses it was concluded that the alkoxyl radical of the hydroperoxide was the initial radical product. Lipid peroxidation is thought to be involved in the aetiologies of various diseases including pre-eclampsia [18, 22, 24]. So far, phospholipid hydroperoxides have been thought to be cytotoxic because phospholipase A2 specifically releases fatty acid hydroperoxides from phospholipids [14, 19]. Transgenic mice over-expressing GPx1 have been found to be partially protected from the lethal effect of anti CD95, underlining the importance of GPx1 and elevated peroxide formation in apoptotic signalling [2, 8]. According to Walsh and Wang, pre-eclamptic trophoblast cells produce about 900 pmol more peroxides than normal cells [23]. We noticed an increased level of MDA and 4-HNA as early 2 hours from the incubation of the *choriocarcinoma* cells with 100 μM

*t*BuOOH. The above results raise the possibility of the participation of endogenous lipid peroxides in the disturbance of hydroperoxide metabolism. On the basis of the results obtained we propose that a naturally occurring rise in hydroperoxides may contribute to the development of pre-eclampsia.

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