Full Length Research Paper

Effect of aqueous ozone solution on pancreatic cells

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The aim of this study was to evaluate the effects of ozone solutions on the viability of pancreatic cells. The ozone was generated by an electrical discharge on high-purity oxygen molecules. Ozonation of the water was performed by bubbling ozone through sterile distilled water (O3 concentration 4 g/1000 L) and saline solution (O3 concentration 5 g/1000 L). After initial dilution of test solutions, serial dilutions were made. In total, seven were tested for both types of solutions (0.00312 mg O3/1000 L, 0.00625 mg O3/1000 L, 0.0125 mg O3/1000 L, 0.025 mg O3/1000 L, 0.05 mg O3/1000 L and 0.1 mg O3/1000 L and 0.2 mg O3/1000 L). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cytotoxicity test was performed in triplicate after 24 h of treatment.

Key words: Ozone, acute pancreatitis, aqueous solution, bactericidal effect.

INTRODUCTION

Ozone is a natural atmospheric compound consisting of three oxygen atoms. It is found in the stratosphere, in the form of a gas with concentrations of 1 to 10 ppm, being continuously created and broken down into molecular O₂ (Amir and Hardy, 2008; Grootveld et al., 2004). Ozone, in the gaseous or aqueous form, has been shown to be a powerful and reliable antimicrobial agent (Arita et al., 2005; Cao et al., 2010). Initially used in the treatment of potable water, ozone is now considered as being one of the most powerful oxidants, with important antiseptic effects, especially on bacteria (Białoszewski et al., 2010). The bactericidal effect of ozone has been documented on a wide variety of organisms, including Gram-positive and Gram-negative bacteria as well as spores and vegetative cells (Guanche et al., 2010). The use of ozonated water has been suggested as an interesting alternative to traditional sanitizers due to its efficacy at low concentrations and short contact time (Bocci, 2007). The oxidant potential of ozone induces destruction of cell walls and cytoplasmic membranes of bacteria and fungi. During this process, ozone attacks glycoproteins, glycolipids, and other amino acids and inhibits and blocks the cell’s enzymatic control system (Amir and Hardy, 2008; Cao et al., 2010). This results increased membrane permeability, the key element of cell viability, leading to immediate functional cessation. Then ozone molecules can readily enter the cell and cause the microorganism to die (Arita, 2005; Guzel-Seydim et al., 2004; Love, 2001). Also, ozone can attack many biomolecules, such as the cysteine, methionine and the histidine residues of proteins (Guzel-Seydim et al., 2004).

Proven antibacterial properties in vitro, ozone is recommended for application in medicine as an adjunct or alternative treatment to combat various local or systemic infections, especially in those cases where traditional therapy has not given satisfactory results (Velano et al., 2001; Białoszewski and Kowalewski, 2003; Celiberti et al., 2006; de Souza et al., 2010; Gu, 2010). Use of ozone in medicine remains controversial because, in high concentrations in gaseous form, the compound has a toxic effect on the human body, more pronounced in the respiratory system. On the other hand, the use of aqueous ozone in different environments, in low concentrations has a beneficial effect on the body both by stimulating host defense mechanisms, and through a direct antimicrobial effect (Oizumi et al., 1998). Thus, aqueous ozone solutions for medical use could be

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administered intraperitoneally, to effectively treat intra-abdominal infection induced in experimental models (Oizumi et al., 1998; Celiberti et al., 2006). Acute pancreatitis with pancreatic necrosis is a severe pathological entity in which the efficiency of standard medical and surgical treatment is limited, poor results obtained by conventional treatment imposes finding on an alternative form of therapy. This paper aims to study the effects of ozone solutions on the viability of pancreatic cells.

**MATERIALS AND METHODS**

**Chemicals**

Culture medium DMEM, fetal calf serum, MTT ((3-(4-5-Dimethylthiazol-2-yl)-2.5 diphenyltetrazolium bromide, Trypan Blue solution were purchased from Sigma–Aldrich, Bucharest, Romania.

**Ozonated water preparation**

For the preparation of ozonated water, a generator developed by Conf. ilie Suaraşan was used. The ozone was generated by an electrical discharge on high-purity oxygen molecules. Ozonation of the water was performed by bubbling ozone through sterile distilled water (O3 concentration 4 g/1000 L) and saline solution (O3 concentration 5 g/1000 L). Ozone concentration in water and in saline solution was measured using an ozonometer (Anseros, Germany).

**Biological materials**

βTC3 cell line was obtained from Oncological Institute (Cluj-Napoca, Romania) (derived from insulinomas generated in transgenic mice carrying a hybrid insulin promoter simian virus-40 tumour antigen gene). The cells were grown in DMEM 4 g/L glucose (Sigma) supplemented with 10% (v/v) fetal bovine serum (Sigma), 2 mM glutamine, 100 UI/ml penicillin, 100 mg/ml streptomycin (Sigma). The cell lines were maintained by serial passage in 25 cm² flasks, incubated at 37°C, in a humidity-controlled incubator at 90% relative humidity and 5% CO₂. Quantification of cells was performed using Trypan-Blue counting chambers using mixed cell suspension in a proportion of 1:2 with Trypan Blue. The cells were counted under a microscope at 100x magnification. The number of viable cells was determined for cell proliferation approach. To evaluate the effect of gaseous ozone on cell viability, cells were treated for 10 min with distilled ozonated water and ozonated saline solution freshly prepared. Results were assessed after 24 h of treatment. Initial concentration of ozone in distilled water was O3/1000 5 g, while saline was 4 g O3/1000 L. After initial dilution of test solutions, serial dilutions were made. In total, seven were tested for both types of solutions (0.00312 mg O3/1000 L, 0.0625 mg O3/1000 L, 0.0125 mg O3/1000 L, 0.025 mg O3/1000 L, 0.05 mg O3/1000 L, 0.1 mg O3/1000 L and 0.2 mg O3/1000 L).

**Viability test**

MTT- cytotoxicity test was performed in triplicate after 24 h of treatment. The cytotoxic effect of the aqueous ozone solution on βTC3 cells was evaluated by tetrazolium reduction assay MTT. Briefly, βTC3 cells seeded in 96-well plate (1x10⁴ cells/well in 200 µL dultecco’s modified eagle’s medium (DMEM), supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 UI/ml penicillin, 100 mg/ml streptomycin), were treated simultaneously with seven concentrations of aqueous ozone solution. Each experimental concentration was tested in triplicate as well. Untreated cells were used as control cells. Plates were incubated at 37°C in 5% CO₂ for 24 h. The supernatants were removed and the cell pellets were washed with phosphate buffer saline (PBS); 200 mL. After 1 h of incubation under standard conditions, the MTT solution was removed from each well. The insoluble formazan resulted, was dissolved in dimethyl sulfoxide (DMSO). Absorbance of the formazan product was measured at 550 nm using a Biotek Synergy HT Microplate Plate Reader. Cell viability was expressed as a percentage of control (cells incubated in normal medium supplemented with distilled water and salt solution).

**Statistical analysis**

Experiments were replicated twice. Measurements were run in triplicate for each replicate (n=2–3). Results were expressed as means±standard deviations. The Student t-test was employed to calculate significance of difference between results. The differences between the means were considered significant at p<0.05. The program used for the statistical evaluation was Origin 7 (OriginLab Corporation, Northampton, USA).

**RESULTS**

βTC3 cells were seeded at 1x10⁴ cells/well in 96 wells microplates, 24 h before treatment. Cell viability was evaluated after Trypan Blue staining and cells were free of bacterial or fungal contamination. βTC3 cells were treated with different concentrations of aqueous ozone solution in DMEM medium for 24 h. Cell viability was expressed as a control percentage (cells incubated in normal medium only), which is considered as 100%. During the MTT test mitochondrial dehydrogenases of living cells reduce the tetrazolium ring, yielding a blue formazan product which can be measured spectrophotometrically. The optical densities obtained are directly proportional to the number of living cells. The cytotoxic effect of a sample is evaluated by the percentage of living cells present in the sample, in relation to the cells treated only with the solvent. After ozonated solution exposure, the culture medium (DMEM 1x) were replaced by a solution of MTT 0.5 mg/ml in Hank’s Buffered Salt Solution (HBSS) medium. After 1 h of incubation, the liquid was aspirated and the insoluble formazan produced was dissolved in DMSO.

The relative cell viability was calculated as percentage respect to the maximal absorbance (vitality = 100×mean OD ozonated cells/mean OD control). After treatment of cells with distilled ozonated water in the highest concentration (0.2 mg) a decrease in cell viability was found (54.98%) with the appearance of an increased number of cells in suspension. Attached cells appear as small clusters that can be given due to changes in their adhesion molecules that appear to be affected by ozone. Higher concentrations of ozone in water solution determined a more evident decrease of viability, up to
54.98%. After treatment of cultures with distilled ozonated water with a concentration of 0.1 mg of ozone the recorded viability was 59.63%. At concentrations of 0.05 and 0.025 mg the cells viability were slightly increased with a reduction of cells in suspension. Cells treated with these concentrations do not show morphological changes. The degree of viability after ozonated water treatment with ozone concentration of 0.0125 and 0.00625 mg was 84.78 and 89.75% (Figure 1).

At the lowest concentration of 0.00312 mg MTT viability test recorded as compared to controls (100%) was 90.37%. BTC3 cells started to have normal form and remained attached on the microplate. Cell viability after ozonated salt solution exposure was assessed by measuring reduction of the tetrazolium dye 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Treatment with ozonated saline solution for 10 min stimulated cell viability depending on the administered concentration. Concentration of 0.00312 mg/ml of saline solution stimulated cell viability (132.86%), as well as for other viable concentrations increased reaching 150% at concentrations of 0.1 mg / ml and 0.2 mg / ml. As shown in Figure 2, no cytotoxic effects were obtained after treatment with ozonated saline. The degree stimulated cell proliferation was dose-dependent. When using concentrations of 0.2 mg / L the percentage of registered viability was 150% compared to the negative control. Global analysis of experimental results indicate statistically significant differences (p<0.05) between the two substances.

**DISCUSSION**

Ozone presents antimicrobial properties (Oizumi et al., 1998; Kim and Yousef, 1999; Paraskeva et al., 1999) that are used for water treatment (Ozmen et al., 1993; Güzel-Seydim et al., 2004) in food industry and treatment of diseases, such as arthritis, otitis and ulcers, pancreatitis, hepatitis. In recent years, there have been reports on the use of German Haslerrail medical ozone therapy system in the treatment of severe hepatitis (Grootveld et al., 2004). The therapy was reported to be effective although its mechanism is not very clear. Unfortunately, the literature shows no data related to the effect of ozone on cell viability. Therefore the purpose of this study focused on evaluating different solutions with different doses of ozone in the degree of proliferation and cell viability. The results of these experiments can be extrapolated and used *in vitro* for evaluation of these solutions in the treatment of pancreatitis. In contrast to the dogma according to which ozone is always toxic, recent studies show that, in certain concentrations, ozone can have disinfectant properties, imunomodulatory effects, can induce antioxidant enzymes, can stimulate metabolism to induce endothelial nitric oxide synthesis, and even stem cell activation, with beneficial effects in neovascularization and tissue recovery (Bocci et al., 2009). Ozone therapy remains a promising field for unconventional medicine, proving its applicability in some diseases and further research will validate the applicability for other types of diseases. Ozonated saline solution can stimulate
Figure 2. βTC3 cell viability after treatment with ozonated saline solution.

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References