Journal of Cell and Animal Biology Vol. 6(2), pp. 25-28, 30 January, 2012
Available online at http://www.academicjournals.org/JCAB
DOI: 10.5897/JCAB11.079
ISSN 1996-0867 ©2012 Academic Journals

Full Length Research Paper

Effect of aqueous ozone solution on pancreatic cells

Al Hajjar Nadim[1], Pitu Flaviu[1], Nicodim Fiț[2], Pitu Florina[1], Popa Calin[1], Suarăsan Ilie[3] and Emoke Pall[2]*

[1]University of Medicine and Pharmacy Cluj-Napoca, Institute of Gastroenterology-Hepatology "Prof. O. Fodor", Romania.
[2]University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine 3-5, Manastur Street, 400372, Cluj-Napoca, Romania.
[3]Technical University of Cluj-Napoca, Romania.

Accepted 19 January, 2012

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, 0.1 mg O3/1000 L and 0.2 mg O3/1000 L). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zoliumbromide (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 1to10 ppm, being continuously created and broken down into molecular O_2 (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

*Corresponding author. E-mail: pallemoke@gmail.com. Tel: +40264596384/ ext.163. Fax: +4026459379.

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 cm^2 flasks, incubated at 37°C, in a humidity-controlled incubator at 90% relative humidity and 5% CO_2. Quantification of cells was performed using Burker-Turk 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, 0.00625 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 ($1x10^4$ cells/well in 200 µL dulbecco'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% CO_2 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 distillated 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 $1x10^4$ 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 distillated 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

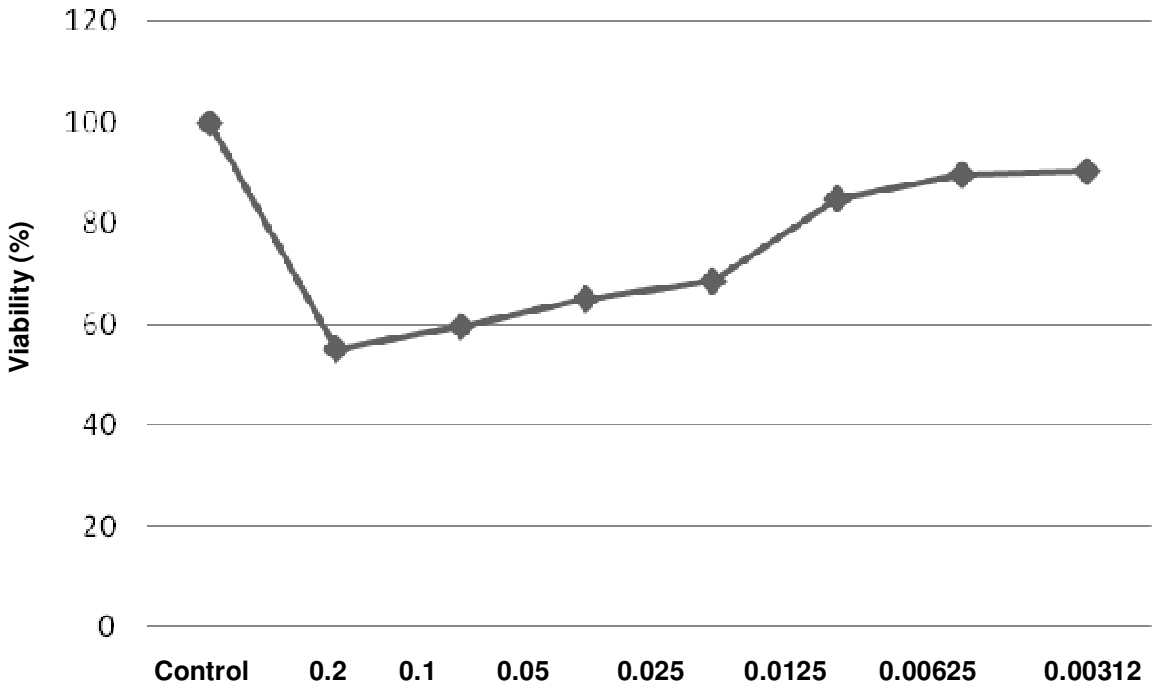

Figure 1. Cells viability after treatment with distillated ozonated water.

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%. βTC3 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

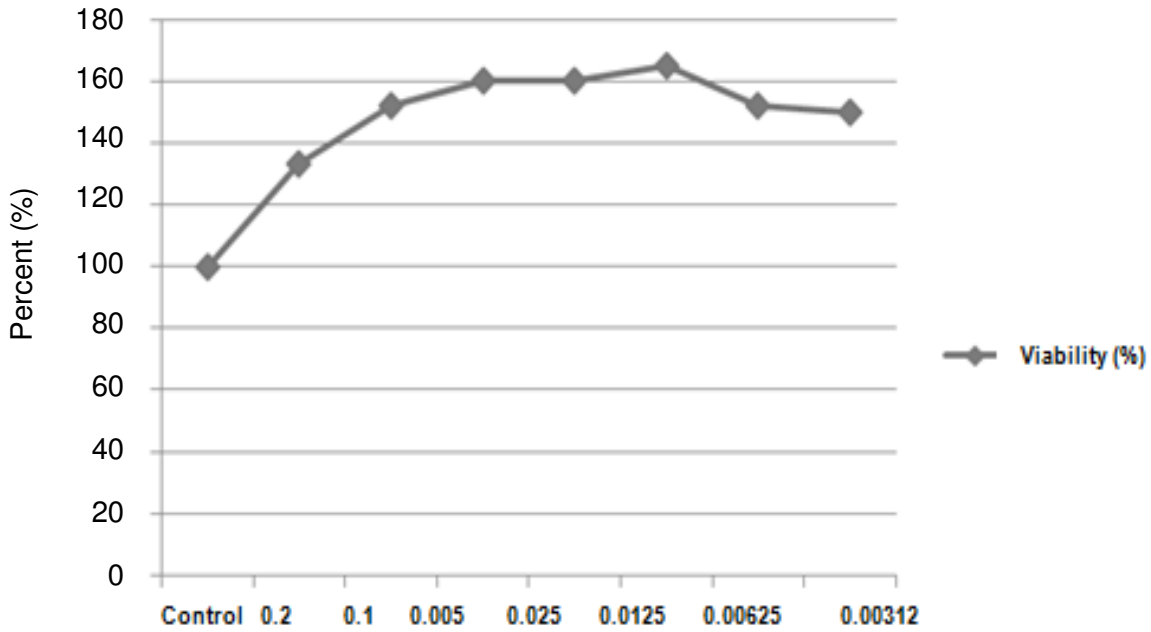

Figure 2. βTC3 cell viability after treatment with ozonated saline solution.

cell proliferation and scarring.

ACKNOWLEDGEMENT

This work was supported by PNCDI - IDEI Nr. 829/2008.

REFERENCES

Amir A, Hardy L (2008). The application of ozone in dentistry: A systematic review of literature. J. Dentistry, 36: 104-116.

Arita M, Nagayoshi M, Fukuizumi T, Okinaga T, Masumi S, Morikawa M (2005). Microbicidal efficacy of ozonated water against Candida albicans adhering to acrylic denture plates. Oral Microbiol. Immunol., 20: 206.

Białoszewski D, Bocian E, Bukowska B, Czajkowska M, Sokół-Leszczyńska B, Tyski S (2010). Antimicrobial activity of ozonated water. Med. Sci. Monit., 16(9): MT71-75.

Białoszewski D, Kowalewski M (2003). Superficially, longer, intermittent ozone theraphy in the treatment of the chronic infected wounds. Ortop. Traumatol. Rehabil., 5(5): 652-658.

Bocci V (2007). The case for oxygen-ozonetherapy. Br. J. Biomed. Sci., 64(1): 44-49.

Bocci V, Borrelli E, Travagli V, Zanardi I (2009). The ozone paradox: ozone is a strong oxidant as well as a medical drug. Med. Res. Rev., 29(4): 646-82.

Cao Rong, Liu Qi, Yin Bang-zhong, Zhu Lan-lan (2010). Combined effect of ozonated water and chitosan on the shelf-life of Pacific oyster (Crassostrea gigas), Innov. Food Sci. Emerg. Technol., 11: 108-112.

Celiberti P, Pazera P, Lussi A (2006). The impact of ozone treatment on enamel physical properties. Am. J. Dent., 19: 67-72.

de Souza YM, Fontes B, Martins JO, Sannomiya P, Brito GS, Younes RN, Rasslan S (2010). Evaluation of the effects of ozone therapy in the treatment of intra-abdominal infection in rats. Clinics, 65(2): 195-202.

Grootveld M, Baysan A, Sidiiqui N, Sim J, Silwood C, Lynch E (2004). History of the clinical applications of ozone. In: Lynch E, editor. Ozone: The revolution in dentistry. London: Quintessence Publishing Co., pp. 23-30.

Gu X-B, Yang X-Y, Zhu H-Y, Xu Y-Q, Liu X-Y (2010). Effect of medical ozone therapy on renal blood flow and renal function of patients with chronic severe hepatitis, Chin. Med. J., 123(18): 2510-2513.

Guanche D, Zamora Z, Hernández F, Mena K, Alonso Y, Roda M, Gonzáles M, Gonzales R (2010). Effect of ozone/oxygen mixture on systemic oxidative stress and organic damage. Toxicol. Mech. Meth., 20(1): 25-30.

Guzel-Seydim Z, Bever PI, Grene AK (2004). Efficacy of ozone to reduce bacterial populations in the presence of food components. Food Microbiol., 21: 475-479.

Güzel-Seydim ZB, Greene AK, Seydim AC (2004). Use of ozone in the food industry. Lebensm Wiss Technol., 37: 453-460.

Kim JG, Yousef AE, Dave S (1999). Application of ozone for enhancing the microbiological safety and quality of foods: A review. J. Food Protect., 62: 1071-1087.

Love RM (2001). Enterococcus faecalis—a mechanism for its role in endodontic failure. Int. Endod. J., 34: 399-405.

Oizumi M, Suzuki T, Uchida M, Furuya J, Okamoto Y (1998). *In vitro* testing of a denture cleaning method using ozone. J. Med. Dent. Sci., 45: 135-139.

Ozmen V, Thomas WO, Healy JT, Fish JM, Chambers R, Tacchi E, Nichols RL, Flint LM, Ferrara JJ (1993). Irrigation of the abdominal cavity in the treatment of experimentally induced microbial peritonitis: efficacy of ozonated saline. Am. Surg., 59(5): 297-293.

Paraskeva P, Lambert SD, Graham NJD (1999). Influence of ozonation conditions on the treatability of secondary effluents. J. Chart Inst. Water E., 13: 430-435.

Velano HE, Nascimento LC, Barros LM, Panzeri H (2001). Avaliação in vitro da atividade antibacteriana da água ozonizada frente ao *Staphylococcus aureus.* Pesq. Odontol. Bras., 15: 18-22.