Ozone Therapy Global Journal vol. 13, nº 1. pp. 47-67, 2023
Official Journal of Aepromo (Spanish Association of Medical Professionals in Ozone Therapy). Madrid, Spain
Publicación Oficial de Aepromo (Asociación Española de Profesionales Médicos en Ozonoterapia). Madrid, España
ISSN: 2174-3215

Original paper

Differential *in vitro* effect of ozone on Neurokinin 1 receptor in both Mesenchymal stem cells and Glioma cells.

Molecular bases of ozone therapy

Juan Carlos Crespo
Department of Pharmacology, Pediatric and Radiology University of Seville. Spain

Sandro Argüelles
Department of Physiology

Mario F. Muñoz
Department of Biochemistry and Molecular Biology

Eugenio Velasco
Department of Stomatology. University of Sevilla.

Miguel Muñoz
Research Laboratory on Neuropeptides (IBIS), Virgen del Rocío University Hospital, Sevilla

Cristina Garzón-Rodriguez
Centre Sociosanitari El Carme. Badalona, Barcelona.Spain

Antonio Ayala
Department of Biochemistry and Molecular Biology

Keywords

stem cells, glioma, adipose derived stem cells; GAMG.

Abstract

Ozone has many therapeutic effects. The mechanism of action of ozone therapy lays mainly in several aspects, being one of them the stimulation of tissue healing. In this work, we have carried out a preliminary study of the possible effect of ozone on the regeneration capacity of tissues. For this, we have studied the effect of ozone on neurokinin 1 receptor (NK1R) levels in mesenchymal stem cells of adipose tissue. NK1R are the cellular receptors of substance P both are involved in cellular proliferation. The results show that ozone specifically increases the expression of NK1R in stem cells but not in cancer cells...

.

Suggestion on how to quote this paper:
Crespo, Juan Carlos et Al. (2023). Differential in vitro effect of ozone on Neurokinin 1 receptor in both Mesenchymal stem cells and Glioma cells.. *Ozone Therapy Global Journal* Vol. 13, nº 1, pp 47-67

Author mail: Antonio Ayala. Departamento de Bioquímica y Biología Molecular. Universidad de Sevilla. Spain. E-mail aayala@us.es
And/Or
Juan Carlos Crespo. Departamento Farmacología, Pediatría y Radiología. Universidad de Sevilla. Spain,. E-mail: doctorcrespodelarosa@gmail.com

Introduction

Ozone is a high reactive gas consisting of three oxygen atoms (O_3). It is useful as a deodorizing and bleaching agent as well as for killing germs and purifying water. It is a pale blue gas at room temperature, and its strong odor is reminiscent of a thunderstorm because lightning strikes produce ozone. Ozone exists in two layers of the atmosphere: stratosphere and troposphere[1]. In stratosphere (the region of the atmosphere between the top of the troposphere and about 50 kilometers altitude), it forms a protective shield against solar ultraviolet radiation. About 90% of atmospheric ozone is contained in the stratospheric "ozone layer". In the troposphere (at ground level and up to 15 km) it is an important greenhouse gas and can be an air pollutant[2]. If all the ozone molecules in the troposphere and stratosphere are brought down to Earth's surface to form a layer of pure ozone over the entire globe, the resulting layer would have a thickness of about three millimeters. This extremely small fraction of the atmosphere plays a vital role in protecting life on earth[3]

The creation and destruction of stratospheric ozone depend primarily on natural processes. The production of stratospheric ozone occurs when ultraviolet light from the sun reacts with oxygen molecules in the upper atmosphere to form the stratospheric ozone layer. When the energetic light strikes oxygen molecules, it breaks them into two separate oxygen atoms, and each of the highly reactive atoms bind with another oxygen molecule, resulting in the formation of two ozone molecules. These reactions occur most frequently over the tropics, where the sunlight is most intense. They are important because the ultraviolet radiation they absorb would otherwise reach the surface of the planet, where it would make it difficult for life to exist.

The production of stratospheric ozone is balanced by its destruction in chemical reactions. Ozone reacts continually with sunlight and a wide variety of natural and human-produced pollutants, mainly nitrogen oxides (NOx) and chlorine derived from chlorofluorocarbons (CFCs) used in refrigerant fluids[4]

Concerning the production of tropospheric ozone, industrial processes are mostly responsible for its creation. It does not have any direct emissions sources, rather it is a secondary gas formed by the interaction of sunlight with components of smog. Smog is made up of many chemicals including nitrogen oxides, Sulphur dioxide, carbon monoxide, volatile organic compounds, particulate matter and ozone itself[5]. Ozone is a primary component of smog. Inside automobile engines, oxygen and nitrogen gas combine to form nitric oxide. This gas reacts with oxygen to form nitrogen dioxide. Ozone levels in the troposphere are not constant. On sunny, hot days, nitrogen dioxide breaks down again to release an oxygen atom, which in turn binds with an oxygen atom to form ozone. Emissions from factories and energy stations that burn fossil fuels also generate ozone through a similar process. Ozone also forms around high-voltage electrical equipment. Tropospheric ozone is a short-lived climate pollutant with an atmospheric lifetime of hours to weeks. Natural levels of ozone are seldom high enough to cause problems for humans, but the extra ozone from industrial processes and automobiles can cause a number of them. The highly reactive gas damages forests and crops, damages living tissue and causes respiratory ailments in sensitive individuals.

Ozone was first suggested as a potable water disinfectant in the nineteenth century because of its powerful ability to inactivate microorganisms. During the first world war doctors applied it topically to infected wounds and discovered O_3 not only remedied infection, but also had hemodynamic and anti-inflammatory properties[6]. Besides its local applications in the treatment of external wounds, ozone therapy has many therapeutic effects[7]. Applied as either a spray, compress or autohemotherapy, O_3 is now used in treating oral infections, circulatory disorders, geriatric conditions, macular degeneration, viral diseases, rheumatism/arthritis, cancer, SARS and AIDS. It also decreases blood cholesterol, reduces oxidative damage, stimulates antioxidative responses, modifies oxygenation in resting muscle and is used in complementary treatment of hypoxic and ischemic syndromes, gangrene, burns and, circulatory disorders, showing to be highly effective in the outcome of these conditions [8].In medical use, the gas produced from medical grade oxygen is administered in precise therapeutic doses, and never via inhalation[9].

The mechanism of action of ozone therapy lays mainly in several aspects: 1.- Immunomodulation o activation of immune system[10]. Ozone increases the production of interferon, tumor necrosis factor and interleukin-2. The production of interleukin-2 launches an entire cascade of subsequent immunological reactions. In addition, it has been described that antibodies catalyze the generation of ozone by a water oxidation pathway [11]. 2.- Inactivation of microorganisms [12-14]. Ozone therapy disrupts the integrity of the bacterial cell envelope through oxidation of the phospholipids and lipoproteins. In fungi, O_3 inhibits cell growth at certain stages. With viruses, O_3 damages the viral capsid and upsets the reproductive cycle by disrupting the virus-to-cell contact with peroxidation. The weak enzyme coatings on cells which make them vulnerable to invasion by viruses make them susceptible to oxidation and elimination from the body, which then replaces them with healthy cells. Ozone was effectively used as an antibacterial agent to treat oral infections [15]. 3.- Stimulation of oxygen metabolism/ antioxidant balance[16]. Ozone has the capacity to oxidize organic compounds [4] to produce peroxides and free radicals giving rise to cascade of reactions like peroxidation of lipids leading to changes in membrane permeability. Because of the formation of lipid ozonation products, there is a stimulation of production of enzymes which act as free radical scavengers and membrane protectors that stimulate tissue repair: glutathione peroxidase, catalase, and superoxide dismutase. Ozone, due to its role as an activator of the antioxidant system make this molecule capable of stimulating tissue healing and has positive effects in the treatment of cardiovascular diseases, improving the patient's prognosis, such as increasing oxygenation in ischemic sites. The hormonal effect of ozone must also be considered [17]

Other effects of ozone include increased endogenous growth factors[18].Ozone therapy also causes an increase in the red blood cell glycolysis rate[6]. This leads to the stimulation of 2,3-diphosphoglycerate which leads to an increase in the amount of oxygen released to the tissues improving oxygenation in the area. Ozone activates the Krebs cycle by enhancing oxidative carboxylation of pyruvate, stimulating production of ATP. It also causes a significant reduction in NADH and helps to oxidize cytochrome C.

As can be seen, the *in vivo* effect of exogenously administered ozone or that produced physiologically, for example, by the immune system, may be due to a direct or indirect action. Depending on the route of administration, the direct effect may not be majority due to the presence of very powerful antioxidants in the serum, such as uric acid, which can limit its half-life in serum. However, its indirect effects such as those described (activator of the immune system or inducer of antioxidant systems, for example) can contribute greatly to its therapeutic effects.

With regard to *in vitro* studies, it is important to bear in mind that there is the difficulty of its permanence in the culture media, which is also short, depending on the composition of the medium and temperature. Perhaps this is the reason why there is no extensive bibliography on them.

Related to these indirect effects, in this work we have carried out a preliminary study of the possible effect of ozone on the regeneration capacity of tissues. We have conducted an *in vitro* study of the effect of ozone on human adipose tissue stem cells (hADSC). To do this, we have investigated the effect of ozonation of culture media on neurokinin 1 receptor (NK1R) levels in ADSC. NK1R plays an important role in the proliferation capacity of these cells [19]. The effect on these cells has been compared with the effect on GAMG cells, which are human glioma cells, to test whether the effect of ozone on NK1R levels is general or cell specific.

MATERIAL AND METHODS

Adipose tissue samples. Human adipose tissue samples were obtained by liposuction surgery and maintained at 4°C. Approval for obtaining human tissue was obtained from a local IRB committee. The patients signed an informed consent form, the result being unanimous approval.

Isolation of stromal vascular fraction (SVF). SVF was obtained as described previously[20]. Briefly, 200–300ml of lipoaspirate was washed, centrifuged and treated with collagenase solution (Collagenase NB 4G, Serva) 0.3U/ml in PBS (Wünsch units). After digestion, an equal volume of Dulbecco's Modified Eagles Medium (DMEM) containing 20% fetal bovine serum (FBS) was added. The mixture was centrifuged at 600xg for 10 min and the pellet was resuspended in 10 ml of expansion medium for non-hematopoietic stem cells (NH- Miltenyi Biotec). Cell suspension was passed through a 100 µm filter (BD Biosciences). The filtrate was centrifuged at 300xg for 10 min. The pellet was resuspended in 5ml of NH medium and passed through a 40 µm filter. Finally, cell numbers were determined in this cell suspension.

Magnetic separation of hADSC. hADSC were magnetically labeled by using CD271 (LNGFR)-APC antibodies and Anti-APC MicroBeads (Miltenyi Biotec)[20]. The cell suspension was centrifuged and the pellet was resuspended in 80ml of rinse buffer (Miltenyi Biotec). Ten ml of CD271-APC antibody were added. After 10min at 4°C, cells were centrifuged at 300x g for 10min.Cells are resuspended with 70ml of buffer rinse and 20ml of Anti-APC Microbeads were added. The samples were incubated for 15 min at 4°C. Cells were washed and centrifuged at 300 x g for 10min. Finally, the pellet was resuspended in 500ml of wash buffer and the sample was passed through the column inserted in the magnetic field according to the manufacturerś instructions.

Cell expansion. After magnetic separation, cells were centrifuged at 350 x g and resuspended in 1ml of NH expansion medium preheated at 37°C. The cells were cultured in a 25 cm^2 flask by adding 4ml of NH Expansion Medium.

Passaging of hADSC. Cell cultures were washed twice with PBS. Enough volume of Trypsin/EDTA (0.05%/0.53 mM) (Gibco) was added to cover the cells and they were then incubated at 37°C for 5 min. Afterwards, DMEM with 20% FBS was added and the sample was centrifuged at 300x g for 10 min at room temperature. The cells were resuspended in 2ml of NH expansion medium.

Phenotyping of hADSC. Phenotyping of ADSC was carried out by flow cytometry and a commercial kit (MSC Phenotyping Kithuman, MiltenyiBiotec) containing the necessary antibodies and fluorophores[20]. The cells were diluted with adipocytes differentiation medium (Miltenyi Biotec). The medium was changed every 3 days. On the21st day, adipogenic differentiation was confirmed by the formation of neutral lipid-vacuoles stainable with Oil Red O (Sigma–Aldrich, USA).

GAMG glioma cell line. The human GAMG glioma cell line (histologically defined as glioblastoma) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine, according to the culture conditions suggested by the American Type Culture Collection (ATCC) and DSMZ. Cells were seeded in 75 cm^2 tissue culture flasks and incubated at 37°C in a 5% CO_2 atmosphere, refreshing the medium every two days.

Immunofluorescence determination in culture cells. Immunofluorescence in cell cultures was determined as described previously [19]. Cells were cultured in a 24-well-plate containing gelatin-coated coverslips. One milliliter of 4% paraformaldehyde was added to each well and the plates were kept for 20 min at 4°C. Wells were washed twice with PBS and incubated with blocking solution (PBS plus 1% fetal bovine serum) for 1 h at room temperature. After removing the blocking solution, each well was washed three times with PBS. Cells were incubated with rabbit-derived anti-NK1R [which specifically corresponds to residues KTMTESSSFYSNMLA, corresponding to the C-terminus of NK1R (amino acids 393–407)] (Sigma, 1:1000). The wells were washed three times with PBS and incubated with the anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (Thermo Fisher) diluted 1:500 in 0.25% Triton X-100 and Hoechst (Sigma) diluted 1: 2000 in PBS for 1 h at 37°C. After incubation, the samples were washed three times with PBS and mounted with mounting fluid for fluorescence (DAKO). Samples were

visualized in a confocal microscope (Zeiss LSM7 DUO). As negative control, the primary antibody was omitted and replaced by non-immune serum. Measurement of the fluorescence intensity of TAC1R was performed using ImageJ software.

Ozone treatment of culture media. In order to carry out this test, the ozone was directly bubbled in 25 ml of culture medium. Because this system produced a lot of foam, a system consisting of two sterile 60 ml syringes faced and joined by a sterile 'T' system was used. In one, 10 ml of medium was placed and the other was filled with 50 ml of ozone gas. Mixing from one syringe to the other was repeated 7 times. In this way a perfect gas-liquid mixture is achieved. After this process, the culture medium was poured over a sterile beaker to measure the amount of ozone. (Picture 1)

Picture1. Ozone treatment of culture media.

Statistical analyses. For statistical analysis the GraphPad Prism Version 5.03 (Graph Pad Software) was used. Statistical evaluation was performed by one-way ANOVA, followed by Tukey's test. A p value of ≤ 0.05 was considered statistically significant.

RESULTS

Solubility and stability of ozone in water

Since in our *in vitro* study ozone was going to be used dissolved in culture media and ozone presents a short shelf life and it is unstable in liquid medium. It was first evaluated the solubility of ozone in distilled water and then compared it with the solubility in culture media. Ozone is partially soluble in water and, like most gases, increases its solubility as the temperature decreases[21]. The study was conducted by bubbling ozone in distilled water at 18°C for 9 min using three different concentrations: (10, 20 and 60) µg/mL. **Figure 1** shows the solubility kinetics over time after the use of these different ozone concentrations.

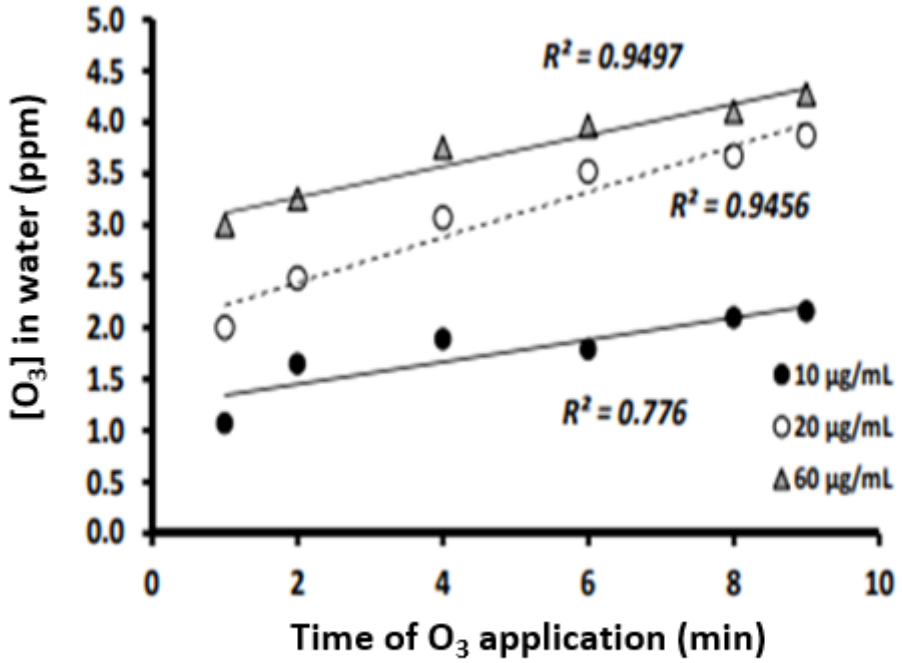

Figure 1.- Ozone solubility in distilled water. The figure shows the concentration of ozone in water over the time of application of ozone gas at three different concentrations (10, 20 and 60 g/ml). Ozone was recorded for time intervals up to 9 min. R is the correlation coefficient for each of the kinetics.

Once the flow of ozone in the water was cut off, the fall in its concentration was observed leaving the ozone meter in water, stirring every 5 minutes to determine the residual value. The results are shown in **Figure 2.** As can be seen, the ozone concentration decays rapidly in the first minute and then, its concentration decays more slowly, with the ozone concentration being proportional to the concentration used in the bubbling.

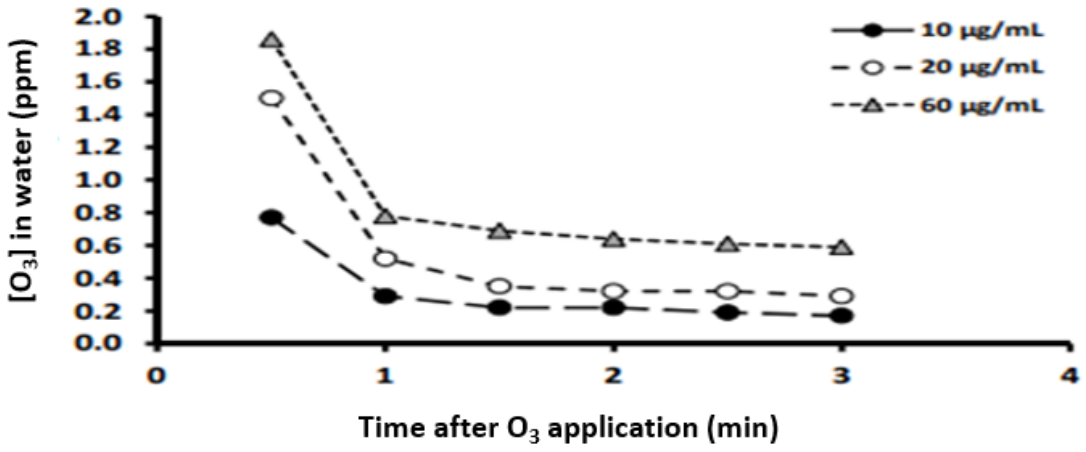

Figure 2.- Residual concentration of ozone in water after bubbling with three different concentrations. The water bubbled with three concentrations of ozone for 9 min. Next, the residual ozone concentration was measured at different times, leaving the ozone meter in water and stirring every 5 min to measure the residual ozone value.

Stability of the ozone in cell culture medium

Since the solubility of ozone depends on the content of organic matter in the liquid (the lower the concentration of organic matter, the longer the half-life of ozone in water) and the cells were grown in ozonated culture medium, we also measure the amount of residual ozone in these media as a function of the temperature. Different concentrations of ozone (10, 40 and 60 µg/ml) were applied. It is observed that with 60 µg/ml ozone lasts much longer in the culture medium and this effect is much greater if the medium has previously been cooled in ice (**Figure 3**)

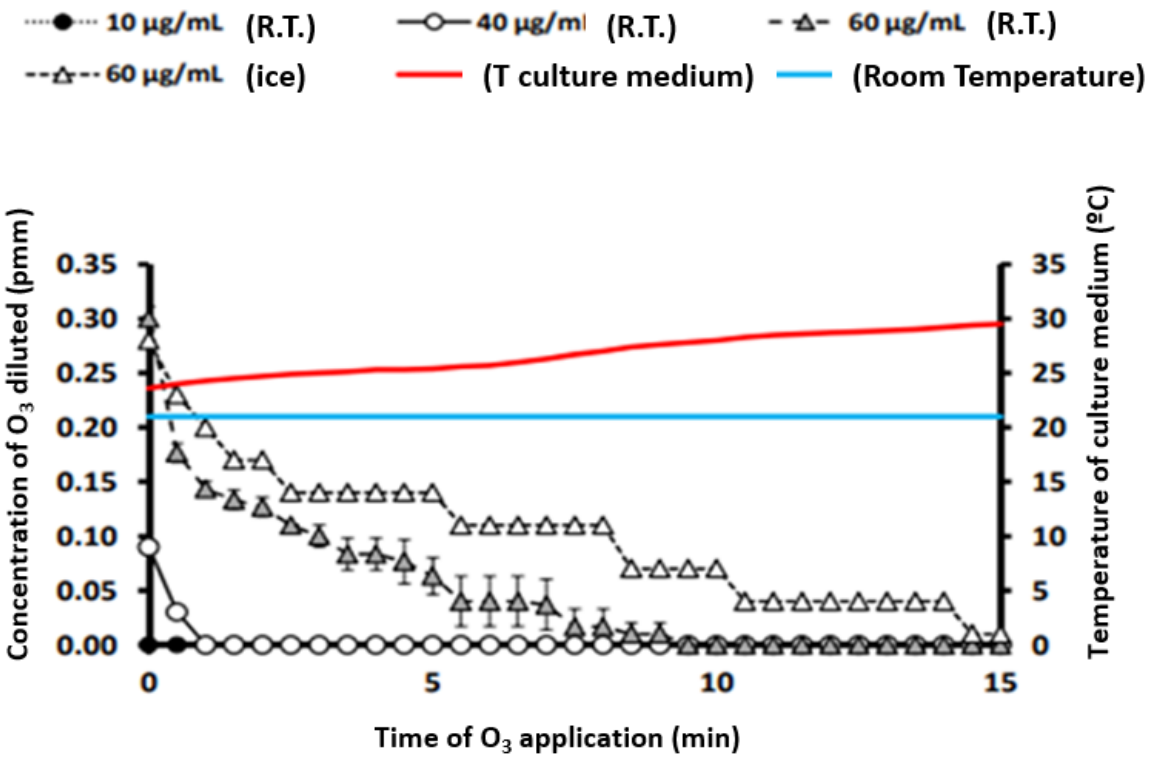

Figure 3.- Changes in the concentration of ozone in the culture medium. Ozone was bubbled in 25 ml of culture medium at room temperature and at 4°C using three concentrations (10, 20 and 60 µg/ml ozone gas)

NK1R localization in human ADSC y GAMG cells

The presence of NK1R was first studied in human ADSC by immunofluorescence (Figure 4).Our results show that these cells expressed the NK1R. Confocal images confirmed that NK1R was located in both nucleus and cytoplasm (**Figure 4 A**). Negative control staining (omitting primary antibodies) showed no evidence of unspecified staining (Non shown results). **Figure 4** also shows the results obtained for the expression ofNK1Rwhen the cells were cultured for different times (30 min, 60 min, 24 and 48 h) in a medium previously treated with ozone (60 µg/ml) by means of two interconnected 50 cc syringes, with applications of 10 times of intercommunication between them with the referred ozone dose and the culture medium in the other.

An increased fluorescence signal corresponding to NK1R can be observed in cells grown (30 and 60) min in ozonated medium (**Fig. 4 A and B**). This fluorescent signal corresponding to NK1R remains increased after 24 and 48 h of culture (**Figure 4 C and D**)

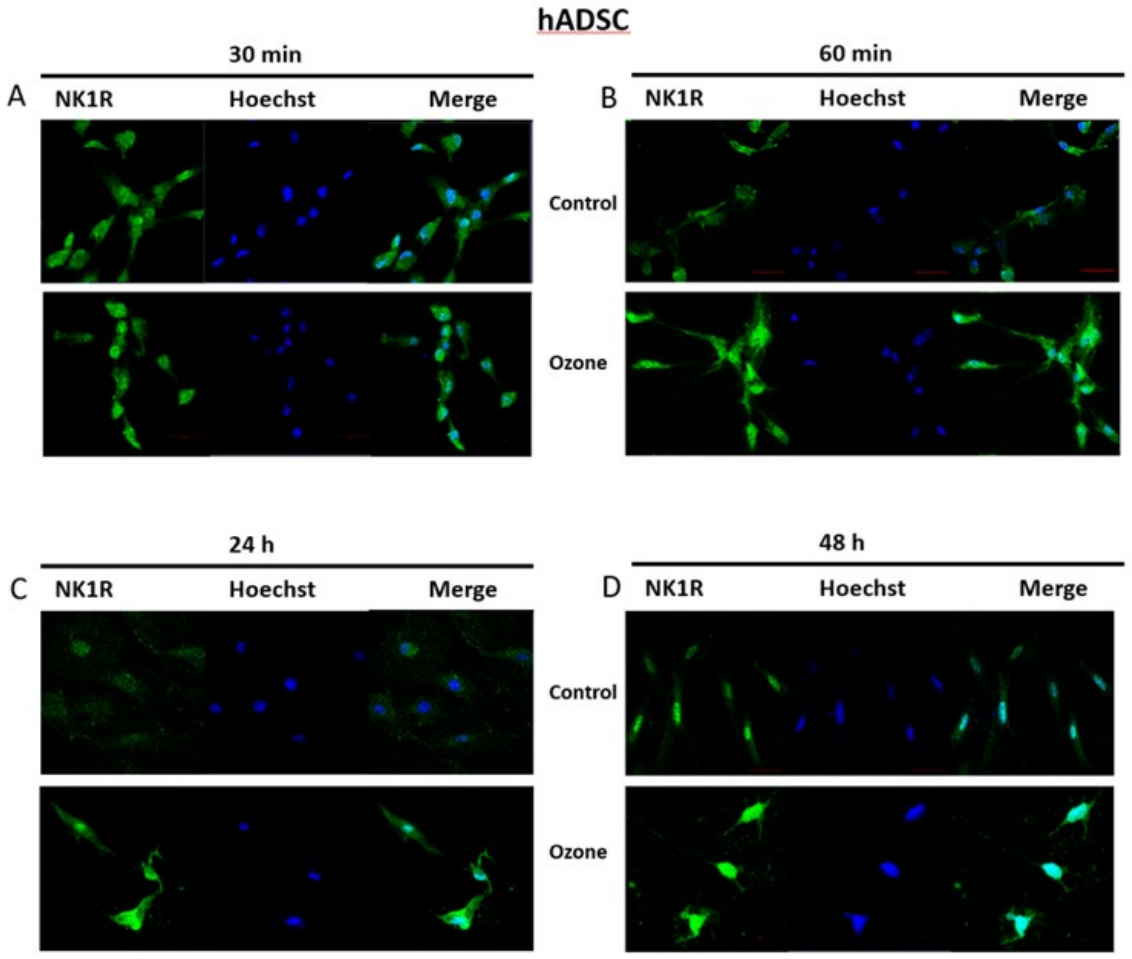

Figure 4. Immunofluorescence localization of NK1R in hADSCs. Localization of NK-1R (green) in hADSC. Nuclei (Blue) are stained by Hoeschst. The results show the immunofluorescence of ADSCs after being cultured at different times (30 min, 60 min, 24 and 48 h) in ozonized culture media.

The quantification of the fluorescent signal at different times is presented in **Figure 5**. It can be observed that at 60 min, there is a significant increase in the expression of NK1R that is maintained at 48 h.

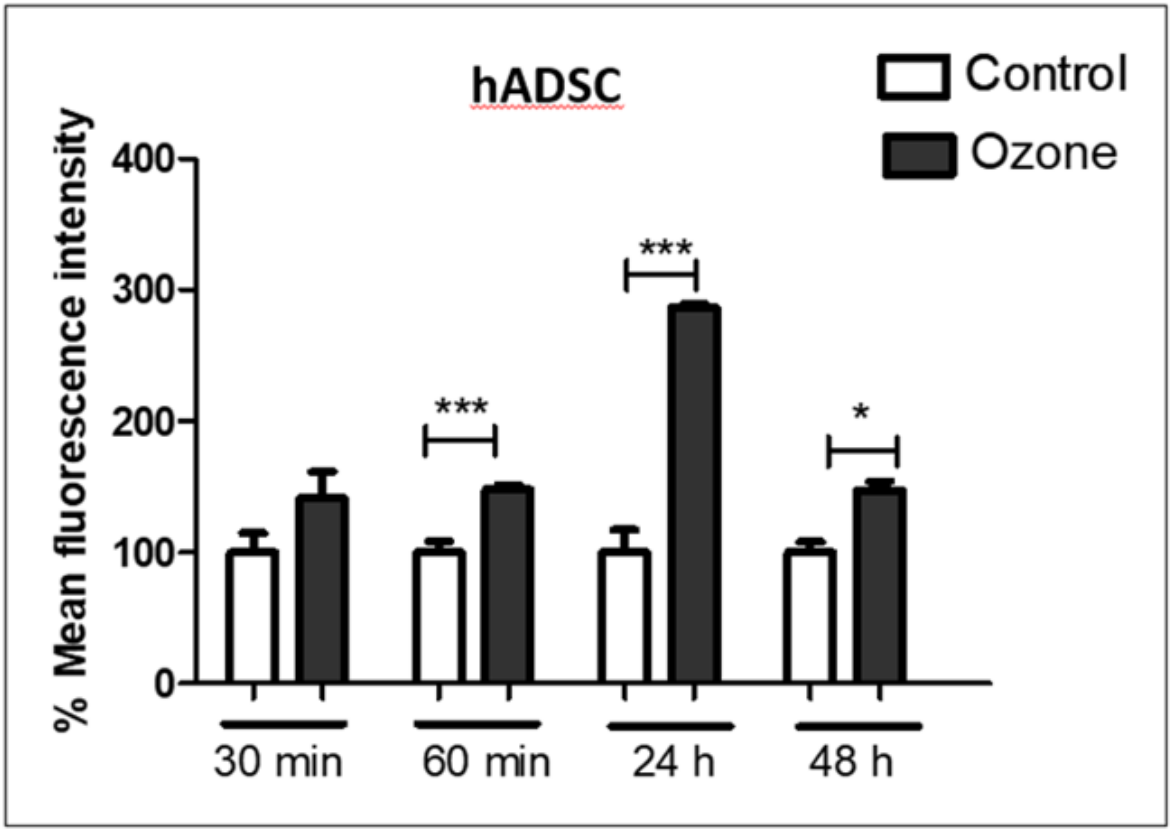

Figure 5. Quantification of the fluorescent signal of NK1R in hADSC. Quantification of the images was carried out as indicated in Materials and Methods section. The results are the average of five experiments +/- SEM.

In the case of GAMG cells, a different effect is observed, since the treatment of the media with ozone does not seem to affect the expression of NK1R (**Figure 6**). These immunofluorescence results were quantified (**Figure 7**). As can be seen, the expression of NK1R did not vary in the GAMG cells when they were cultured in an ozonated medium at any of the times analyzed. Even, there is a decrease in the expression at 60 min.

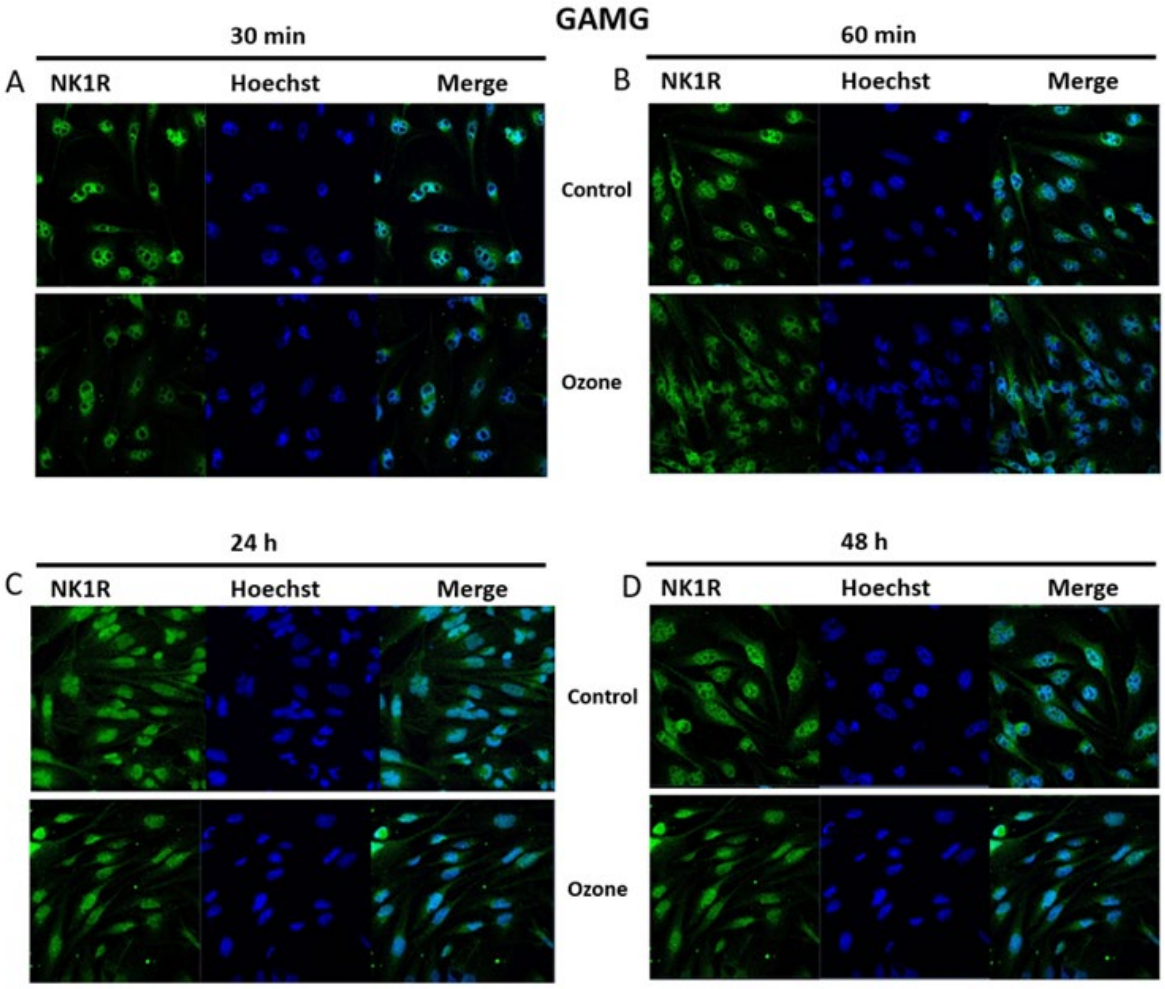

Figure 6. Immunofluorescence localization of NK1R in GAMG cells. Localization of NK-1R (green) in hADSC. Nuclei (Blue) are stained by Hoeschst. The results show the immunofluorescence of ADSCs after being cultured at different times (30 min, 60 min, 24 and 48 h) in ozonized culture media.

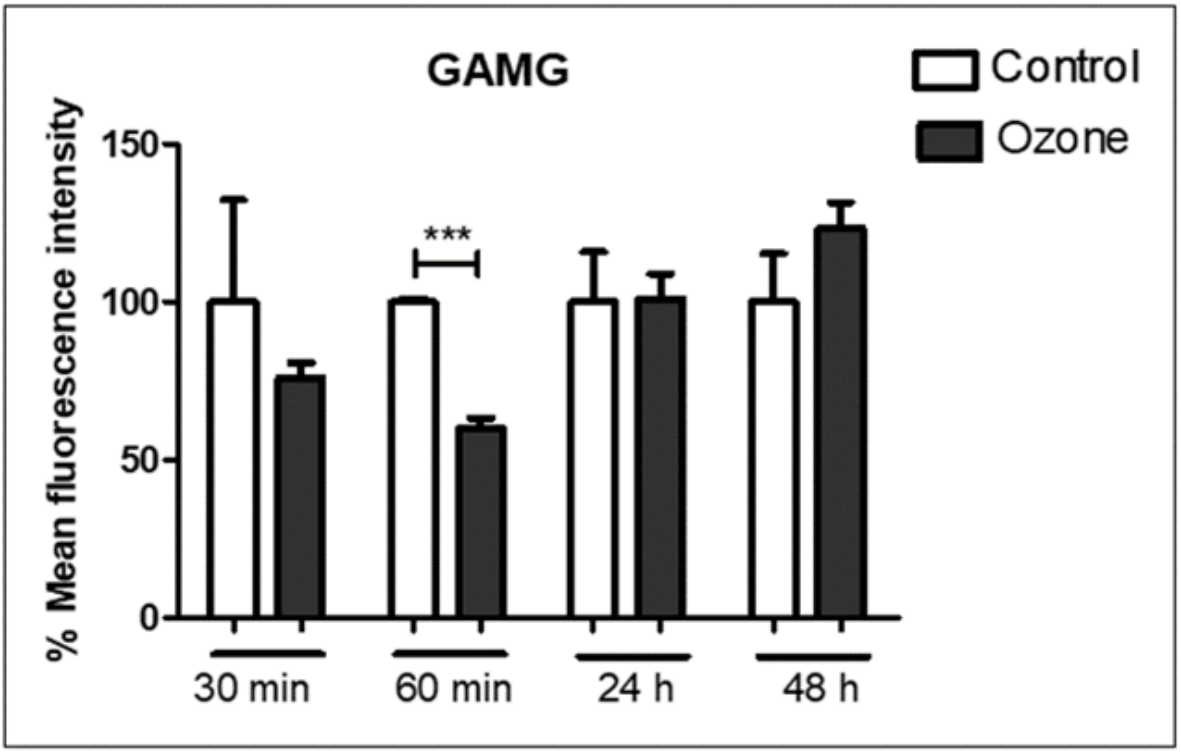

Figure 7. Quantification of the immunofluorescence images shown in Figure 6. Quantifications were performed as indicated in Material and Methods. The results are the average of five experiments +/- SEM.

DISCUSION

In this paper we study the effect of ozone on NK1R levels in mesenchymal stem cells of adipose tissue. The rational is to know what short-term effect the ozonation of culture media can have on their possible capacity for tissue regeneration.

Adipose tissue has proven to serve as an abundant, accessible, and rich source of mesenchymal stem/stromal cells (MSCs)[22]. These cells have multipotent properties suitable for tissue engineering and regenerative medical applications, including potential restoration of organ optimal functions that decline during aging[23, 24]. Once they are isolated from adipose tissue, they have to be characterized by checking that 1.- they express the mesenchymal antigens CD73, CD90 and CD105, 2.- they do not have hematopoietic antigens such as CD34, CD45, CD14 and CD11b,and 3.- they are able to differentiate into adipocytes, osteoblasts and chondrocytes.
Stem cells can be classified in the following groups according to their potential and differentiation capacity [25]:
1. Totipotents: only the zygote and the results of the first 2 divisions are totipotent cells, since they have the ability to form both the embryo and the trophoblast of the placenta.

2. Pluripotent: after 4 days, the totipotent cells begin to differentiate and form the blastocyst and the internal cell mass. The cells of the latter are considered pluripotent and can differentiate into the 3 germlines (endoderm, mesoderm and ectoderm), but lose the ability to form the placenta.
3. Multipotents: they are cells capable of producing a limited range of lineages of differentiated cells according to their location. For instance, stem cells of the central nervous system have the potential to generate 3 cell types: neurons, oligodendrocytes and astrocytes.
4. Unipotent: are cells capable of generating a single specific cell type; for example, stem cells in the basement membrane of the interfollicular epidermis, which produce only keratinized scales.
Stem cells have unique characteristics: they have the capacity for self-renewal and differentiation into different cellular types, contributing to the regeneration of damaged tissues.
Due to the high multipotential capacity, plasticity and actions of mesenchymal stem cells, they are of great interest for clinical application[25]. These cells have been used inthe repair of the myocardium, bone, and field of ophthalmology for the treatment of corneal ulcers and retinal degenerations. On the other hand, promising results have been obtained in the area of general surgery in the treatment of fistulas from different locations such as the respiratory and digestive tract. In addition, important studies are being carried out in diabetes mellitus, neurodegenerative diseases and liver regeneration.

It has been proposed that regenerative cell therapy provides not only stem cells, but also various active and regulatory molecules produced or induced by them, with the ability to favor the differentiation of implanted cells and stimulate the cells of the host tissue. This would contribute to a physiological regeneration of damaged tissue[25, 26]. Their immunomodulatory effects are also important: due to the immunomodulatory capacity of these cells, they can avoid allogeneic rejection through different mechanisms. Numerous studies have shown that mesenchymal stem cells do not allow antigen recognition by interfering with the function of dendritic cells and T lymphocytes, so they have a local immunosuppressive effect due to their ability to secrete cytokines. This effect is enhanced when cells are exposed to a medium with inflammatory activity or characterized by the presence of high levels of interferon gamma. The cellular and molecular mechanisms by which ADSC exert their therapeutic action are still under investigation and work is continually appearing in relation to their phenotypic and functional characterization and how to improve it.

In relation to ozone and its therapeutic use, there are several mechanisms described [27]One of them is that it increases tissue regeneration, the result being dependent on the type of tissue to which it will be applied. Precisely, to deepen its stimulating effect on the ability to regenerate, we have studied the effect of ozone on ADSCs. In our experiments, ozone was applied at different concentrations in the culture media prior to the use of these media to grow hADSCs.

First, we have established ozone concentration and temperature conditions to ozonate the media. The results show that the solubility of ozone in water and culture media is proportional to the amount of ozone used and the time of application (Fig. 1). After applying, the amount of ozone decays rapidly and this decay is less if the culture media are ozonated at 4^0C. The decay of ozone in distilled waters is characterized by a fast initial decrease of ozone, followed by a second phase in which ozone decreases by first order kinetics[27], with hydroxyl radicals (OH) being the main products of this decomposition.

Due to that ozone can react with organic compounds in aqueous solution by a direct ozone reaction or by an indirect reaction of OH radicals, formed from the decomposition of ozone, we study the solubility and stability after the bubbling of ozone in the culture media used for cells. This study allowed us to determine the optimal ozone concentration, as well as the time of use of the ozonized media after ozone treatment and the optimal ozonation temperature.

A molecule is a dipole (as in H_2O) when there is an asymmetric distribution of electrons because the molecule is made up of atoms of different electronegativity. As a consequence, the electrons are preferentially in the vicinity of the more electronegative atom. This creates two regions (or poles) in the molecule, one with a partial negative charge and the other with a partial positive charge. When two polar molecules (dipoles) approach each other, an attraction is produced between the positive pole of one of them and the negative pole of the other.

A particularly interesting example of dipole-dipole interactions is hydrogen bonding. The dipole moment (m) is a vector (oriented towards the negative charge and whose magnitude depends on the intensity of the charge and the distance between the atoms) that allows the asymmetry of charges in the molecule to be quantified. The shape of the molecule also affects the dipole moment. In the example of water, one hydrogen is joined by hydrogen bonds and the other by the dipole-dipole forming a bond by Van der Va alls Force, that curiously they break at a body temperature of 36º C, which the temperature used in our work.

Next, we focus on the effect of ozonation of culture media on hADSCs. Specifically, we studied whether ozone affects the expression levels of NK1R receptors, which are the cellular receptors of substance P. Substance P (SP) is a neuropeptide belonging to the tachinid peptide family[28]. SP, after binding to its receptor, the neurokinin 1 receptor (NK1R), controls several transcription factors such as NF-kB, hypoxia inducible factor (HIF-1a), c-myc, c-fos, c-jun, and AP-1. SP and NK1R have a widespread distribution in both the central and peripheral nervous systems. We have previously demonstrated the presence of different isoforms of NK1R that have different subcellular locations in the ADSC[19]. NK1R immunoreactivity was much higher in the nucleus than in the cytoplasm. SP induces proliferation and mitogenesis through NK1R in ADSCs.We have described that SP induces proliferation and mitogenesis through NK1R in ADSCs. Taken together, these findings could suggest a role for SP/NK1R systems in the cytoplasm and nucleus functions of the ADSCs such as proliferation, differentiation and migration. Our previous results also show a positive correlation between DNA concentration and NK1R expression, suggesting the possible involvement of nuclear NK1R in the mitogenesis process.

This means that the NK1R could regulate the nuclear activity of stem cells and that SP could act either as genetic or epigenetic nuclear factor to regulate gene expression. The increased expression of NK1R in the nucleus of ADSC during mitosis over the levels found in non-dividing cells demonstrated that this receptor plays a pivotal role in the genetic cell cycle machinery of stem cells.

In this context, the results suggest that ozone seems to contribute to the regenerative capacity of stem cells. These cells are quiescent most of the time and their cell cycle is activated when it is necessary to regenerate a tissue.

This effect of ozone on the regenerative capacity of tissue can be combined with other mechanisms of action described by other authors[17], such as:a) it improves blood circulation and oxygen delivery to ischemic tissue owing to the concerted effect of NO and CO and an increase of intraerythrocytic 2,3-DPG level;b) by improving oxygen delivery, it enhances the general metabolism;c) it upregulates the cellular antioxidant enzymes and induces HO-1 and HSP-70;d) it induces a mild activation of the immune system and enhances the release of growth factors from platelets;e) it procures a surprising wellness in most of the patients, probably by stimulating the neuro-endocrine system.

To see if the effect of ozone on hADSC is specific or general, we have also studied the effect of ozone in the expression of NK1R in human glioma cell line (GAMG) cells. As can be seen, these cells also express the NK1R receptor (Fig. 6). The presence of the NK1R in these cells agrees with the data reported in previous studies on the localization of the receptor in the nucleus/cytoplasm of other human cancer cells (e.g., melanoma, gastric and colon carcinomas, breast cancer, lung cancer) [29-32]

Our results show that unlike hADSC, ozone did not increase receptor expression. This differential effect suggests that ozone may have a regenerative effect by stimulating the proliferation of hADSC but would not supposedly have any effect of activating the cell division of glioma cells in the event that there was some tumor process mediated by these cells in progress. It remains to be determined whether this same effect can be extended to other cell types, but the idea is that ozone selectively stimulates the expression of certain cell types and not in all, so a worsening in cancer patients treated with ozone could not be expected. On the contrary, a regenerative effect of healthy tissues could be expected. That is to say that you could not expect an enhanced malignant potential.

In conclusion, we can say that the concentration of ozone in the medium of pluripotent mesenchymal stem cells seem to increase their mitotic capacity and in theory their regeneration capacity. This effect appears to be mediated by increased expression ofNK1R. This activation by ozone therapy is dose dependent. This NK1R is selective for the peptide neurotransmitter substance P, a molecule of intercellular communication, promoter of pain and neurogenic inflammation, and activator of the fibroblast. Although these results can be considered as preliminary, NK1R seems to be a relevant element of the molecular mechanisms of the action of ozone therapy, which could be used for the treatment of the pathologies in which substance P is participating.

On the other hand, ozone does not appear to have an effect on GAMG cells, suggesting that the possible stimulating effect on the regeneration capacity of ADSCs is not accompanied by indiscriminate activation of cell proliferation of normal and cancer cells.

References

1. Rowland, F.S., *Stratospheric ozone depletion.* Philos Trans R Soc Lond B Biol Sci, 2006. **361**(1469): p. 769-90.
2. Möller, D., *The tropospheric ozone problem.* Arh Hig Rada Toksikol, 2004. **55**(1): p. 11-23.
3. Ross J. Salawitch, D.W.F., Michaela I. Hegglin, Laura A. McBride, Walter R. Tribett, Sarah J. Doherty,, *Twenty Questions and Answers About the Ozone Layer: 2018 Update, Scientific Assessment of Ozone Depletion: 2018.* 2019.
4. *Environmental effects of ozone depletion and its interactions with climate change: Progress report, 2016.* Photochem Photobiol Sci, 2017. **16**(2): p. 107-145.
5. Ebi, K.L. and G. McGregor, *Climate change, tropospheric ozone and particulate matter, and health impacts.* Environ Health Perspect, 2008. **116**(11): p. 1449-55.
6. Elvis, A.M. and J.S. Ekta, *Ozone therapy: A clinical review.* J Nat Sci Biol Med, 2011. **2**(1): p. 66-70.
7. Smith, N.L., et al., *Ozone therapy: an overview of pharmacodynamics, current research, and clinical utility.* Med Gas Res, 2017. **7**(3): p. 212-219.
8. Gulmen, S., et al., *Ozone therapy as an adjunct to vancomycin enhances bacterial elimination in methicillin resistant Staphylococcus aureus mediastinitis.* J Surg Res, 2013. **185**(1): p. 64-9.
9. Bocci, V., *How Is Ozone Administered?* OZONE: A new medical drug, 2010: p. 27-33.
10. Bocci, V. and N. Di Paolo, *Oxygen-ozone therapy in medicine: an update.* Blood Purif, 2009. **28**(4): p. 373-6.
11. Lerner, R.A. and A. Eschenmoser, *Ozone in biology.* Proceedings of the National Academy of Sciences, 2003. **100**(6): p. 3013.
12. Polydorou, O., et al., *The antibacterial effect of gas ozone after 2 months of in vitro evaluation.* Clin Oral Investig, 2012. **16**(2): p. 545-50.
13. Cattel, F., et al., *Ozone therapy in COVID-19: A narrative review.* Virus Res, 2021. **291**: p. 198207.
14. Korzun, W., J. Hall, and R. Sauer, *The effect of ozone on common environmental fungi.* Clin Lab Sci, 2008. **21**(2): p. 107-11.
15. Domb, W.C., *Ozone therapy in dentistry. A brief review for physicians.* Interv Neuroradiol, 2014. **20**(5): p. 632-6.
16. Re, L., *Molecular Aspects and New Biochemical Pathways Underlying Ozone Effects.* Clinical and Experimental Pharmacology, 2018. **8**: p. 1-4.
17. Bocci, V.A., I. Zanardi, and V. Travagli, *Ozone acting on human blood yields a hormetic dose-response relationship.* J Transl Med, 2011. **9**: p. 66.
18. Zhang, J., et al., *Increased growth factors play a role in wound healing promoted by noninvasive oxygen-ozone therapy in diabetic patients with foot ulcers.* Oxid Med Cell Longev, 2014. **2014**: p. 273475.
19. Muñoz, M., M.F. Muñoz, and A. Ayala, *Immunolocalization of Substance P and NK-1 Receptor in ADIPOSE Stem Cells.* J Cell Biochem, 2017. **118**(12): p. 4686-4696.
20. Muñoz, M.F., et al., *Effect of Age and Lipoperoxidation in Rat and Human Adipose Tissue-Derived Stem Cells.* Oxid Med Cell Longev, 2020. **2020**: p. 6473279.
21. Egorova, G.V., et al., *Ozone Solubility in Water.* Moscow University Chemistry Bulletin, 2015. **70**(5): p. 207-210.
22. Zuk, P.A., et al., *Human adipose tissue is a source of multipotent stem cells.* Mol Biol Cell, 2002. **13**(12): p. 4279-95.
23. Al-Ghadban, S., M. Artiles, and B.A. Bunnell, *Adipose Stem Cells in Regenerative Medicine: Looking Forward.* Frontiers in Bioengineering and Biotechnology, 2022. **9**.
24. Jung, J.S., et al., *Adipose-Derived Stem/Stromal Cells Recapitulate Aging Biomarkers and Show Reduced Stem Cell Plasticity Affecting Their Adipogenic Differentiation Capacity.* Cell Reprogram, 2019. **21**(4): p. 187-199.
25. Si, Z., et al., *Adipose-derived stem cells: Sources, potency, and implications for regenerative therapies.* Biomedicine & Pharmacotherapy, 2019. **114**: p. 108765.

26. Weiss, A.R.R. and M.H. Dahlke, *Immunomodulation by Mesenchymal Stem Cells (MSCs): Mechanisms of Action of Living, Apoptotic, and Dead MSCs.* Front Immunol, 2019. **10**: p. 1191.
27. von Gunten, U., *Ozonation of drinking water: part I. Oxidation kinetics and product formation.* Water Res, 2003. **37**(7): p. 1443-67.
28. Muñoz, M. and R. Coveñas, *Involvement of substance P and the NK-1 receptor in human pathology.* Amino Acids, 2014. **46**(7): p. 1727-50.
29. Muñoz, M., A. González-Ortega, and R. Coveñas, *The NK-1 receptor is expressed in human leukemia and is involved in the antitumor action of aprepitant and other NK-1 receptor antagonists on acute lymphoblastic leukemia cell lines.* Invest New Drugs, 2012. **30**(2): p. 529-40.
30. Muñoz, M., et al., *The NK-1 receptor is expressed in human melanoma and is involved in the antitumor action of the NK-1 receptor antagonist aprepitant on melanoma cell lines.* Lab Invest, 2010. **90**(8): p. 1259-69.
31. Muñoz, M. and R. Coveñas, *Involvement of substance P and the NK-1 receptor in pancreatic cancer.* World J Gastroenterol, 2014. **20**(9): p. 2321-34.
32. Munoz, M. and R. Covenas, *NK-1 receptor antagonists: a new generation of anticancer drugs.*Mini Rev Med Chem, 2012. **12**(7): p. 593-9.