

Apoptosis Regulation in Dental Pulp Cells and PD-1/PD-L1 Expression Dynamics Under Ozone Exposure – A Pilot Approach

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Abstract

This study aimed to determine the effect of ozone on the expression of *Bax* and *Bcl-2* genes in dental pulp cells. Additionally, the programmed cell death protein 1, programmed death-ligand 1, and CD200 antigens were determined in lymphocytes to assess their surface expression. Dental pulp cells were cultured from extracted healthy third molars and characterized as dental pulp stromal cells. Gene expression of *Bcl-2* and *Bax* was analyzed at 0 s, 6 s, and 12 s of ozone exposure using real-time PCR. Lymphocytes from dental pulp were subjected to ozone exposure for 12 s and PD-1, PD-L1, and CD200/CD200R expression was analyzed by flow cytometry. Upon exposure to ozone for 6 s, the *Bcl-2* expression decreased significantly to -0.09 , and at 12 s, it increased significantly to 0.3 . *Bax* gene expression level increased significantly to 0.188 after 6 s exposure, and at 12 s, to 0.16 . Lymphocytes exposed to ozone for 12 s showed minimal changes in PD-1, PD-L1, and CD200/CD200R expression levels, indicating that oxidative stress does not impact the signaling pathways regulating these molecules. The significant upregulation of *Bcl-2* at 12 s highlights the cells' effort to protect themselves from prolonged oxidative stress, possibly tipping the balance toward cell survival and tissue repair. However, the absence of changes in PD-1 and PD-L1 expression on lymphocytes under oxidative stress suggests that these molecules are not sensitive to oxidative stress in this context.

Keywords

Ozone • Apoptosis • Dental pulp cells • CD19⁺ B lymphocyte • CD4⁺ T lymphocyte • CD8⁺ T lymphocyte

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1. Introduction

Ozone, composed of three oxygen atoms (O_3), is the most potent oxidant found in nature. It is generated by lightning and solar ultraviolet radiation. The pharmacological properties of ozone are due to its unique molecular structure (Sen and Sen 2020). Ozone reacts with various biomolecules, including lipids, polyunsaturated fatty acids, and amino acids. Its involvement in lipid peroxidation leads to the formation of products such as singlet oxygen, hydrogen peroxide, and hydroperoxides (Bocci and Aldinucci 2004; El Meligy et al. 2023). These agents may interact with other cellular components, potentially generating harmful substances (Shamim 2018).

Owing to the strong antioxidant capacity of the blood, which comprises both hydrophilic and lipophilic antioxidants along with cellular enzymes, a portion of the ozone dissolved in the plasma is swiftly neutralized by antioxidants like uric acid, ascorbic acid, reduced glutathione, cysteine, and albumin.

The leftover ozone predominantly reacts with polyunsaturated fatty acids (PUFAs) found mainly in the three hydrophobic domains of albumin (Travagli et al. 2010). Reactive forms of oxygen, such as ozone, or substances derived from their reactions with lipids, proteins, or nucleic acids, may affect living organisms in many different ways. First of all, they can cause structural disruptions in DNA. This results in the occurrence of various kinds of point mutations, like base pair rearrangements, deletions, and insertions. Moreover, chromosomal alternations may occur, leading to mutations in tumor-suppressor genes and, as a result, allowing for the expression of the mutated phenotype (Renaudin 2021). Ozone stimulates both the humoral and cellular immune systems through the activation of pathways linked to the transcriptional factors, nuclear factor of activated T cells, and activated protein 1, which subsequently triggers the transcription of genes associated with cytokines. Therefore, there is an increase in the production of interferon (IFN)- γ , interleukin (IL)-2, and tumor necrosis factor (TNF)- α (Smith et al. 2017; Di Mauro et al. 2019).

Ozonated autohemotherapy is particularly effective for treating vascular diseases such as stroke, peripheral arterial disease, and chronic heart disease. Although it can be used as an adjunctive therapy for chronic infectious diseases, diabetes, and cancer, it is not a cure for these conditions. Conversely, topical application is highly effective for treating cutaneous and ulcerative infections. The gaseous

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oxygen-ozone mixture is remarkably important in orthopedics (e.g., herniated disk). The application of ozone in the medical field has been indicated for preventing and treating various pathologies in dentistry. Dental caries, gingivitis, periodontal diseases, and oral mucosal lesions, mainly caused by bacteria, viruses, and fungi, can benefit from ozone treatment. Ozone therapy is also used in dentistry to treat oral lesions e.g., herpes labialis, aphthous ulcers, and premalignant disorders like oral lichen planus. Ozone facilitates natural healing in extraction wounds and gingival infections. Ozonated water might be used to manage oral infectious organisms in dental plaque so it is the strategy for reducing dental cavities. Ozone has been proposed as an important option in the treatment of dental caries in primary and permanent teeth. Some of the studies prove that ozone affects open caries, non-cavitated occlusal carious lesions, pit and fissure caries, and primary root caries. In halitosis, ozone can help to lessen bad breath by destroying the microorganisms. In endodontics, ozone is used effectively to disinfect root canals during endodontic treatment (El Meligy et al. 2023; Goswami et al. 2024; Veneri et al. 2024).

Ozone leads to the destruction of organisms primarily by damaging the cytoplasmic membrane of cells as a consequence of ozonolysis and secondly by inducing changes in the intracellular contents due to secondary oxidant effect leading to protein oxidation and loss of organelle function (Sen and Sen 2020). However, it has not been explained fully till now in the literature how ozone causes cell injuries. The above-mentioned lipid peroxidation is one of the possible theories. It is claimed that ozone reacts with cholesterol and phospholipids present in cell membranes. As a result, oxysterols (ozonized cholesterol products) appear in cells. These substances can generate reactive oxygen species (ROS) and be responsible for causing DNA damage, inducing mutations and apoptosis. However, whether ozone itself can reach the cell fluids is still under discussion (Cataldo 2006; Kosmider et al. 2010).

Apart from oxysterols, ozone induces the creation of 4-hydroxy-2-nonenal (HNE). HNE is a harmful aldehyde that predominantly interacts with amino acids such as cysteine, lysine, and histidine. It is cytotoxic, but its action is dose-dependent. In low concentrations ($<0.1 \mu\text{M}$), it stimulates enzyme activity such as phospholipases. In high concentrations ($1\text{--}100 \mu\text{M}$), it causes cytotoxic, genotoxic, and carcinogenic effects. Additionally, it induces apoptosis or even necrosis if the concentration is $>100 \mu\text{M}$ (Diomedea et al. 2022). Despite its detrimental effects, ozone has been used as a therapeutic agent. Its antibacterial properties were known and widely used during the First World War for curing infected wounds and poison gas injuries. This ozone property makes it an attractive disinfecting agent used, for example, for drinking water sterilization (Bocci et al. 2011). Additionally, its degradation technology, with promising potential, has

been the focus of numerous studies in recent decades. Special ozone therapy, called ozone oxidative preconditioning, is used to prepare the organism for toxic effects mediated by oxidative stress.

One of the theories states that a specific dose of ozone is capable of generating enough hydrogen peroxide to trigger cellular response to oxidative stress. As a result, antioxidant enzymes appear in the cytoplasm and reduce H_2O_2 , thereby reducing the likelihood of further generation of ROS (Bocci and Aldinucci 2004). Oxidative stress is a harmful response characterized by elevated levels of ROS within cells. Numerous previous studies have examined the impact of oxidative stress on pulp cells. Some studies showed that the oxidative stress of human dental pulp cells promotes the reduction of odontoblastic capability. The dynamic processes within mitochondria are crucial for preserving the balance of the dental pulp's internal environment. Inflammation and oxidative stress can trigger changes in mitochondrial dynamics, leading to cell death in the dental pulp (Shirawachi et al. 2022; Vaseenon et al. 2023).

Another proposed mechanism of the protective action of ozone involves the suppression of apoptosis by restoring the Bcl-2 protein level (Chen et al. 2008). *Bcl-2* is an anti-apoptotic gene originally found to be overexpressed in human B-cell lymphoma (Dissanayaka et al. 2020). It is situated on the outer membrane of mitochondria, where it has a notable role in preserving cell survival and counteracting the activities of pro-apoptotic proteins. Further research has shown its influence on additional factors such as cell differentiation, growth, and angiogenesis. The proangiogenic activity of Bcl-2 is implicated in its anti-apoptotic ability, by interfering with the function of a proangiogenic factor Bcl-associated X (Bax) protein and subsequent activation of caspases. Moreover, Bcl-2 activates angiogenic CXC chemokines, such as CXCL1 growth-related oncogene- α (GRO- α), and CXCL8 (IL-8). Its function, along with the Bax protein, is to determine the susceptibility of cells to apoptosis (Rak et al. 2000).

Programmed cell death protein 1 (PD-1), also referred to as CD279, is a cell surface receptor expressed in human T cells, B cells, monocytes/macrophages, and some cancer cells. Along with its ligand programmed death-ligand 1 (PD-L1), PD-1 is crucial for negatively regulating T-cell immune function stability and integrity. This helps prevent T-cell overactivation that could lead to autoimmune responses (Ghosh et al. 2021). PD-1 is a 288-amino acid protein composed of an N-terminal IgV domain, a transmembrane domain, a cytoplasmic tail with two tyrosine-based signaling motifs, and a 20-amino acid sidechain separating the IgV domain from the plasma domain. PD-L1 and PD-L2, the ligands of PD-1, are both type 1 transmembrane glycoproteins containing IgC and IgV domains (Yi et al. 2022).

CD200, formerly known as OX-2, is a transmembrane glycoprotein that transmits a negative immunoregulatory signal to

dampen inflammatory reactions and promote immune tolerance. Expression of CD200 in T cells, both at the mRNA and protein level, is regulated by TNF- α and IFN- γ (Jin et al. 2021). Dental pulp stromal cells (DPSCs) isolated from oral tissues possess long-term proliferation ability and multipotency properties that are exploited for clinical purposes, including tissue regeneration and immunomodulation (Marconi et al. 2023). Multiparameter flow cytometry of immunophenotyping is a quick and effective method to evaluate and correlate various cellular characteristics such as size, internal complexity, and antigen expression within a cell population simultaneously.

This study aimed to determine the effect of two exposure times of ozonation on the expression of *Bcl-2* and *Bax* genes in dental pulp cells. Dental pulp cells were cultured in a medium saturated with ozone for either 6 s or 12 s. Concurrently, the study evaluated the percentage of lymphocytes expressing PD-1 and PD-L1 antigens to assess their surface expression.

2. Materials and Methods

The study was submitted to, and approved by, the Ethical Committee at the Medical University of Lublin All volunteers (Poland) under the Number KE-0254/53. All volunteers signed an informed consent form before any sample collection.

2.1. Dental pulp cells culture

Human dental pulp was obtained from sound, extracted permanent teeth (third molars) without carious lesions or restorations. The sample consisted of 30 teeth. The teeth were extracted from healthy individuals aged 18–25 years. Each sample was assigned a random number and all tests were conducted by trained, masked observers. After extraction, the teeth were cleaned from the remaining connective tissue and debris. Subsequently, they were disinfected and rinsed with distilled water. Then, an experienced operator prepared each tooth using a sterile, calibrated bur in a high-speed handpiece with air-water-cooling. The following procedures were performed on each sample: a longitudinal furrow was created using a safe-end flame-shaped diamond bur (Meisinger, Germany) ensuring no contact with the pulp tissue. Then the teeth were sectioned using a dental elevator, and the pulp tissue was

gently separated with a sterile dental excavator. The time between tooth extraction and pulp removal was minimized; the pulp was immediately removed and immersed in the culture medium. Within a maximum of 30 min, it was transported to the laboratory in polystyrene microtubes. The dental pulp tissue was minced into small pieces, approximately 1 mm in size, and digested with an enzyme solution (3 mg/mL collagenase type I and 4 mg/mL dispase) in phosphate-buffered saline for 45 min at 37°C with gentle agitation to digest the tissue. After digestion, the tubes were centrifuged at 1500 rpm for 5 min to pellet the cells and the cell pellet was resuspended in fresh Dulbecco's Modified Eagle Medium (Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA), 1% penicillin/streptomycin (Sigma-Aldrich, USA). The cell suspension was filtered through a 70 μ m cell filter to remove any undigested tissue fragments and the cells were seeded into 100-mm cell culture dishes with the growth media, at 37°C and 5% CO₂.

2.2. Methods of ozone application

Ozone generation in the liquid cell culture was achieved using the Ozotop device (TIP TOP TIPS Sarl, Switzerland) specifically the Ozotip CORO model. This is an apparatus with an open cycle of O₃ and can produce ozone at 700 ppm and ozone efficiency of 1 L/min.

In the experiment, the ozone was applied to 20 mL of liquid cell culture for durations of 0 s, 6 s, and 12 s. Isolated lymphocytes were subjected to ozonation for 12 s only. Subsequently, the conductivity of the liquid cell culture was measured using the waterproof conductivity meter CC 101 (Elmetron, Poland) to confirm the appropriate ozonation of the medium (Table 1).

2.3. *Bax* and *Bcl-2* genes expression

After the cell culture was completed, total RNA from the cells was isolated using a modified Chomczyński and Sacchi's method (Chomczynski and Sacchi 1987), employing the following reagents: TRI Reagent (Sigma), chloroform (Sigma), isopropanol (Sigma), 75% ethanol (Poch, Poland). The concentration and purity of RNA were assessed using a spectrophotometric method (NanoDrop 2000c, Thermofisher Scientific,

Table 1. Medium conductivity (mS) depending on time of ozonation and time after ozonation

Ozonation time (s)	Conductivity measurement after ozonation (mS) Average of 5 measurements, deviation \pm 1 mS		
	Immediately after ozonation	After 24 h of keeping medium in 4°C	After 48 h of keeping medium in 4°C
6	12	11	9
12	15	12	10

USA). Based on isolated RNA, cDNA synthesis reaction was performed. Specifically, 1 µg RNA was dissolved in water and High-Capacity cDNA Transcription Kits with RNase Inhibitor (Applied Biosystems, USA). The reaction was conducted in a volume of 20 µL following the manufacturer's protocol, in a thermal cycler under/in the following conditions: 10 min 25°C, 120 min 37°C, 5 min 85°C. Subsequently, the obtained cDNA was replicated with a PCR real-time method in a volume of 25 µL, employing the StepOnePlus system (Applied Biosystems). The reaction mixture comprised: 1 µL cDNA, 1.25 µL of TaqMan probe (Bax Bcl GAPDH, Applied Biosystems), 12.5 µL of TaqMan Gene Expression Master Mix buffer (Applied Biosystems), and 10.25 µL of ultrapure water.

The reaction was conducted according to the scheme: 10 min of denaturation in 95°C and then 40 two-stage cycles: 95°C – 15 s, 60°C – 60 s. Endogenous reaction control constituted the GAPDH reference gene. The PCR reaction proceeded as follows: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of a two-stage process: denaturation at 95°C for 15 s, and extension at 60°C for 60 s. The GAPDH reference gene served as the endogenous reaction control. The control sample utilized was a control colony labeled "without ozone." The average of relative samples expression was calculated using Expression Suite Software Version 1.0.3 (Applied Biosystems).

2.4. Isolation of lymphocytes for PD-1 and PD-L1 expression analysis

The entire tooth pulp was collected immediately after tooth extraction. Lymphocytes were isolated directly from dental pulp obtained from extracted human teeth. Lymphocytes were isolated according to the accepted procedure on gradient L and were subjected to ozonation for 12 s, as described above, and then subjected to immunophenotyping for the presence of PD-1 and PD-L1. Cells before ozonation constituted the control group.

2.5. Immunophenotyping

Immunophenotyping was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm argon laser. Briefly, cells after ozone application (treated cells) or cells not treated with ozone, constituting the experimental control, were stained with monoclonal antibodies (purchased from BD Biosciences) conjugated with different fluorochromes to determine the proportion of the specific cell types and the surface expression of PD-1 and PD-L1. Percentages of PD-1-positive and PD-L1-positive T and B lymphocytes were determined using combinations of the following monoclonal antibodies: CD45/FITC, CD14/PE, CD3/CyChrome, CD19/FITC, CD4/FITC, CD8/FITC,

CD279 (PD-1)/PE, and CD274 (PD-L1)/PE. A minimum of 1000 events were acquired. Additionally, the percentages of cells expressing specific surface markers were determined. All FACS data were analyzed using the CellQuest Software (BD Biosciences). An example of cytometric analysis is presented in Figure 1.

2.6. Statistical analysis

The research results were subjected to statistical analysis and the values of the analyzed variables were presented using mean and standard deviation. The normality of the distribution of variables in the study groups was assessed using the Shapiro–Wilk normality test. To examine differences between "before and after ozonation" measurements, the Student's *t*-test for dependent samples or ANOVA for multiple groups was employed. The *p*-value <0.05 was considered statistically significant. All data are described as mean ± SD in the text. The database and statistical tests were conducted using Statistica 9.1 computer software (StatSoft, Poland).

3. Results

3.1. Isolation success rate and cells morphology

A total of 30 teeth were included in the study. In the study, we used the human dental pulp tissue of permanent teeth. *In vitro* culture was positive for 6 teeth. The remaining 24 samples showed either no cell growth or contamination during long primary culture. About 17 days after the beginning of culture, cells that have expanded successfully reached about 90% confluence on the plate and showed a fibroblast-like, elongated morphology.

3.2. The expression of *Bcl-2* and *Bax* genes in dental pulp cells exposed to ozone

As apoptosis-inducing agents frequently signal by modifying the expression of Bcl-2 family proteins, we have chosen to explore potential alterations in *Bcl-2* and *Bax* genes in dental pulp cells after ozone exposure. The cells were exposed to ozone for varying durations (6 s and 12 s), and the relative expression levels (logRQ) of these genes were measured and compared to control cell cultures not subjected to ozonation (0 s). The average relative expression of the *Bcl-2* gene in cell culture not subjected to ozonation (0 s) was 0.057. Upon exposure to ozone for 6 s, the *Bcl-2* expression decreased significantly to –0.09 and increased significantly to 0.3 at 12 s (Figure 2). The significant upregulation of *Bcl-2* at 12 s suggests a strong anti-apoptotic response to longer ozone exposure. This implies that dental pulp cells activate

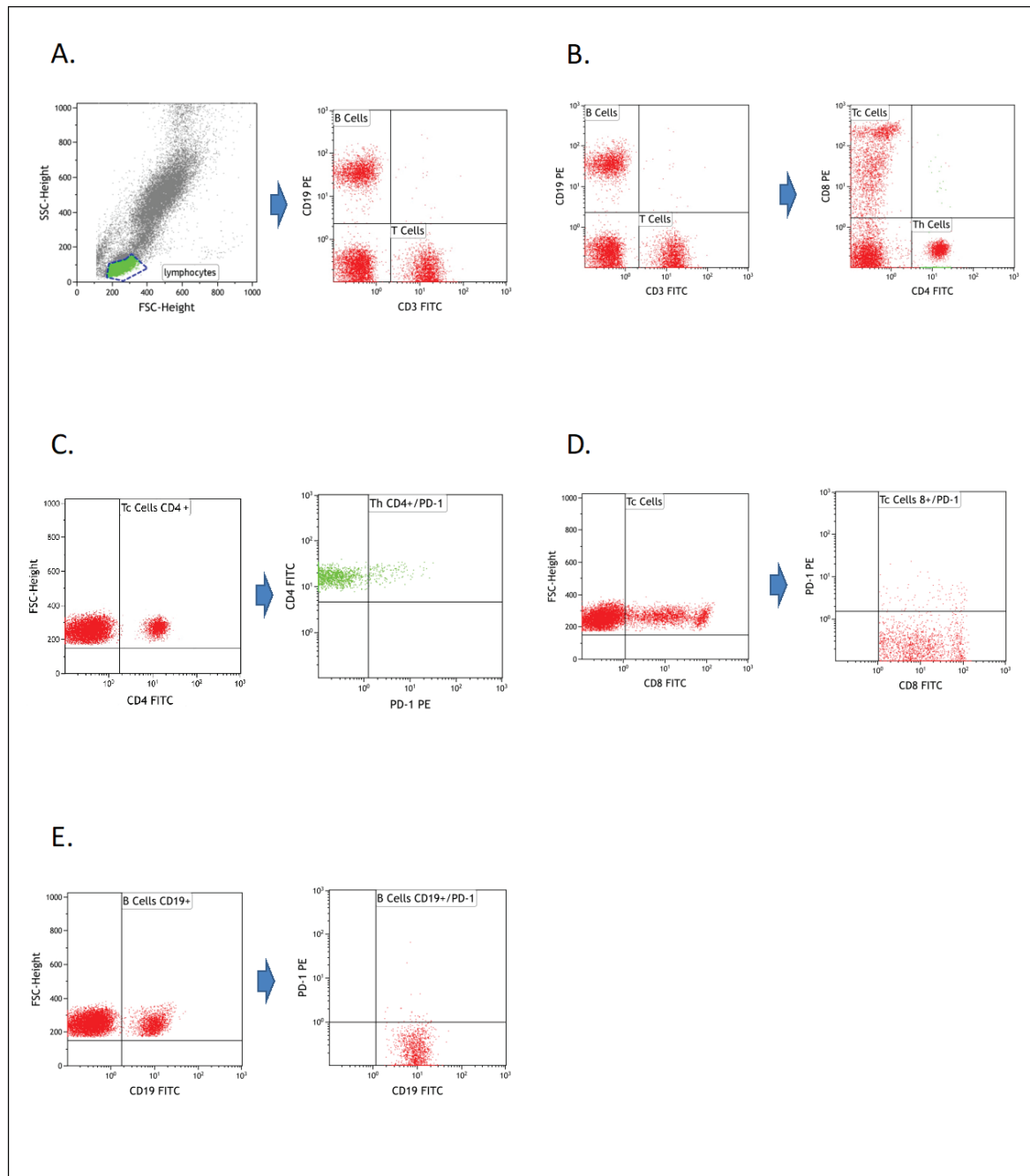


Fig 1. An example of the flow cytometry analysis of antigens expression on T and B lymphocytes. **(A)** Gating strategy for lymphocytes as well as their subpopulations, B lymphocytes (CD19+) and T lymphocytes (CD3+); **(B)** Gating strategy for Tc (CD8+) and Th (CD4+) lymphocytes among T cell subpopulations (CD3+); **(C)** Strategy for gating PD-1 expression among the Th cell population; **(D)** Gating strategy for PD-1 expression among the Tc cell population; **(E)** Gating strategy for PD-1 expression among the B cell population. PD-1, programmed cell death protein 1.

survival mechanisms to counteract potential oxidative stress induced by ozone.

Similarly, the *Bax* gene showed a notable change in expression with ozone treatment for 6 s. The relative expression in the not ozonated group (0 s) was 0.0048. After 6 s of

ozone exposure, the expression level increased significantly to 0.188, and at 12 s, to 0.16 (Figure 2). The moderate upregulation of *Bax* at both 6 s and 12 s indicates a pro-apoptotic response of the cells to ozone exposure. However, the lack of further increase at 12 s suggests a

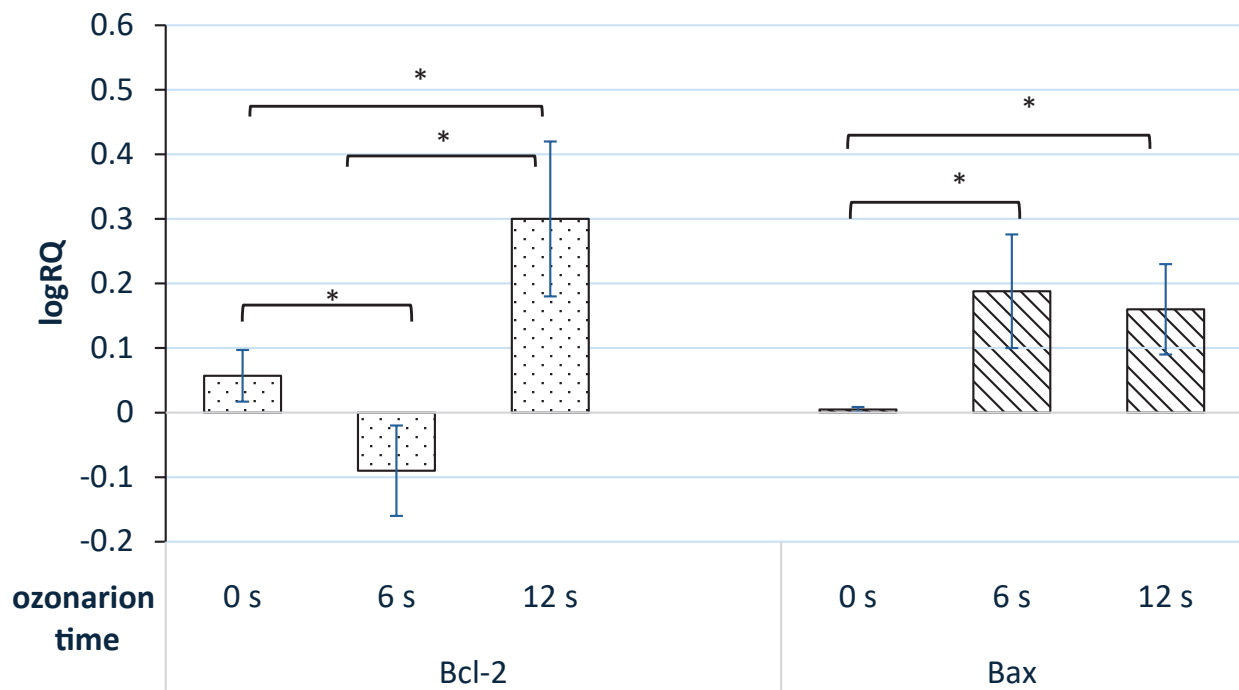


Fig 2. Average relative expression of *Bcl-2* and *Bax* genes (logRQ) in dental pulp cells cultures depending on the ozonation time compared to cells without ozone treatment. The cells not subjected to ozonation (ozone dose 0 s.) or exposed to ozone for 6 s or 12 s were analyzed for the expression of *Bax* and *Bcl-2*. Data are representative of six independent experiments, each repeated in duplicate \pm SD; *Indicates significant difference between two indicated groups ($p < 0.05$, ANOVA test).

Table 2. *Bax/Bcl-2* expression ratio in response to ozonation of dental pulp cells

Ozonation time (s)	<i>Bax</i> mean (logRQ)	<i>Bcl-2</i> mean (logRQ)	<i>Bax/Bcl-2</i> ratio	<i>p</i> -value
0	0.0048	0.0573	0	0.199
6	0.1883	-0.0903	-2	0.0011*
12	0.1611	0.3	0.5	0.0005**

The relative expression levels of the *Bax/Bcl-2* ratio in dental pulp cells exposed to different ozonation times. The ratio of *Bax/Bcl-2* was used to represent anti-apoptotic efficiency.

*Indicates a difference between 0 s, 6 s, and 12 s treatments ($p = 0.05$).

**Represent statistically significant differences between 6 s and 12 s treatment at $p = 0.05$.

plateau in the pro-apoptotic signaling, allowing for a controlled cellular response.

The *Bax/Bcl-2* ratio is a crucial indicator of cell fate, reflecting the balance between apoptosis and survival. Next, the balance between pro-apoptotic (*Bax*) and anti-apoptotic (*Bcl-2*) signals within dental pulp cells under different ozonation times was measured (Table 2). Overall, the analysis of *Bax/Bcl-2* ratio analysis indicated a dynamic response of dental pulp cells to ozone exposure. At 6 s of ozonation, the strong relative expression of *Bax* compared to *Bcl-2* was observed, suggesting a tilt toward pro-apoptotic signaling, which may be a response to the initial oxidative stress induced by ozone exposure. The higher regulation

of *Bcl-2* and the mild regulation of *Bax* after 12 s of exposure to ozone suggest a strong anti-apoptotic response, likely reflecting the cells' efforts to survive and counteract prolonged oxidative stress.

3.3. The expression of immune regulatory molecules PD-1, PD-L1, CD200, CD200R in lymphocytes

It is well known that ozonation can induce oxidative stress in various cell types, including lymphocytes. Consequently, excessive oxidative stress caused by ozonation may lead to apoptosis of lymphocytes. This could impact the total count of lymphocytes in the sample, potentially leading to a decrease in their numbers.

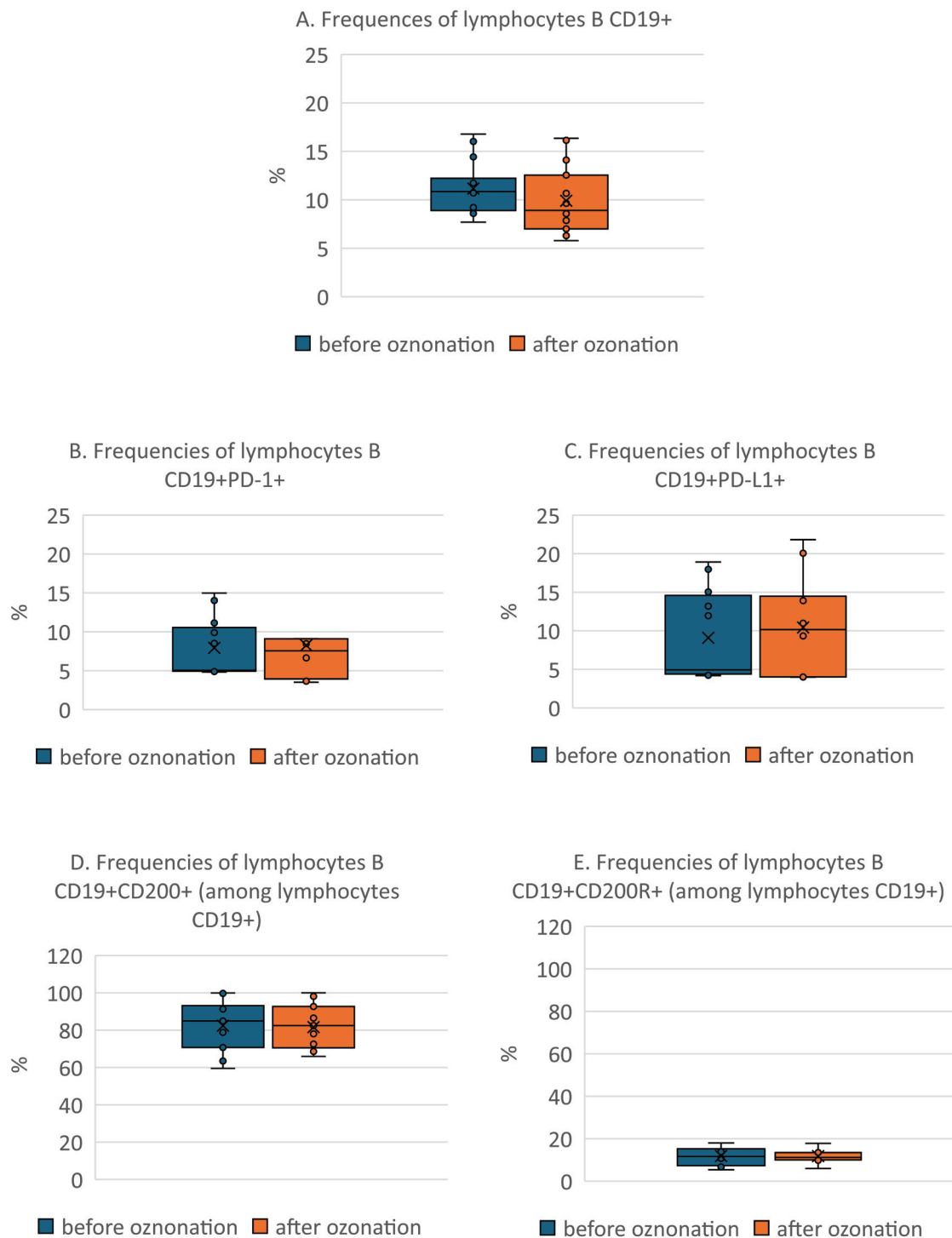


Fig 3. Differences in B lymphocyte measurements before and after ozonation. The percentages of CD19+ B lymphocytes (A) expressing PD-1 (B) and PD-L1 (C) molecules in the studied cell populations isolated from dental pulp of healthy donors (n=15) before and after ozonation. CD19+CD200 expression (D) and CD19+CD200R expression in B lymphocytes (E) before and after ozonation. Dots represent the cases (n = 15), (—) median, (x) mean, whiskers indicate quartiles. PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1.

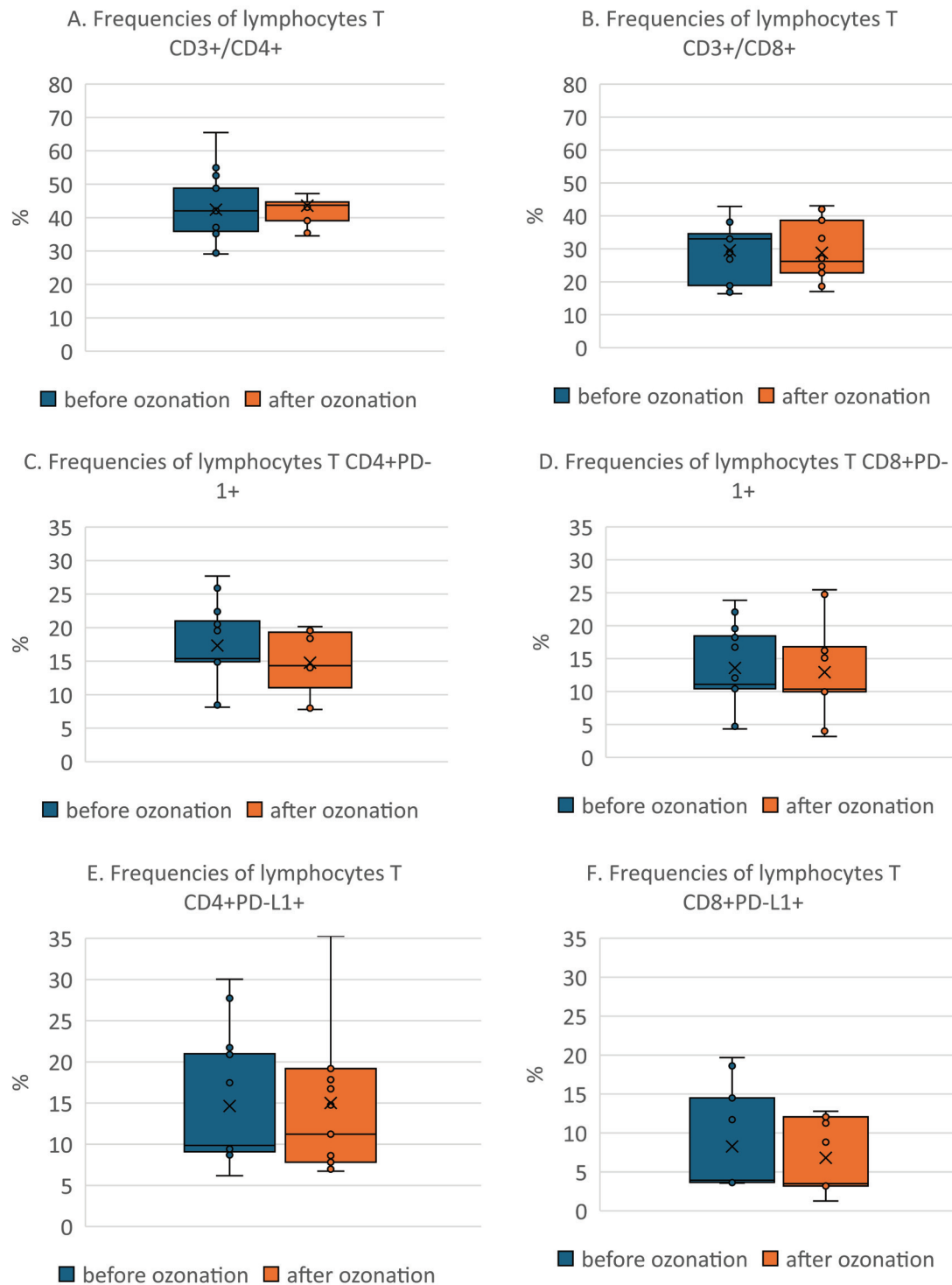


Fig 4. Differences in T lymphocyte measurements before and after ozonation. The percentages of CD4+ (A) and CD8+ (B) T lymphocytes expressing PD-1 (B, E) and PD-L1 (C, F) molecules in the studied cell populations isolated from dental pulp of healthy donors (n = 15) before and after ozonation. Dots represent the cases (n = 15), (—) median, (x) mean, whiskers indicate quartiles. PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1.

In the next step, lymphocytes from dental pulp were subjected to ozonation for 12 s and then their frequency was compared to the control group. We observed no notable alterations in the frequencies of Th, Tc cells and B cells, between the two groups (Figures 3a, 4a, and 4b). However, the average frequency of B cells and Th cells were slightly decreased in ozonated samples compared to the controls.

Considering a possible correlation between ozonation and the percentages of lymphocytes expressing PD-1 and PD-L1 molecules, a statistical analysis was applied, indicating that despite some minor changes, ozonation does not significantly influence the expression of PD-1 and PD-L1 on B (Figures 3b and 3c) or T (Figures 4c, 4d, 4e and 4f) lymphocytes.

The interaction between CD200 and CD200R generally results in the suppression of immune responses. Oxidative stress-induced changes in the expression of these molecules can enhance this suppression, contributing to an immune regulatory environment. In our experiment, CD200 and CD200R expression levels on B lymphocytes were unaffected by oxidative stress (Figures 3d and 3e), indicating that oxidative stress does not impact the signaling pathways regulating these molecules. This could be due to a lack of interaction between oxidative stress signaling pathways and the regulatory elements controlling CD200/CD200R expression. The stability of analyzed molecules may also imply that the immune regulation and immune suppression mechanisms involving these molecules are robust and maintain their function despite oxidative challenges.

4. Discussion

Ozonotherapy has become an integral component of infection treatment across various medical specialties including dermatology, oncology, and surgery. Its application in dentistry has been recognized for preventing and treating various pathologies such as dental caries, gingivitis, periodontal diseases, and oral mucosal lesions, primarily caused by bacteria, viruses, and fungi. Ozone primarily acts on microbial cells by damaging their cytoplasmic membrane, which destroys these microorganisms. Over the past decade, numerous researchers have investigated the potential mechanisms of action triggered by ozone in dental procedures (Tang et al. 2005; Sies 2015; Szymona et al. 2019). Ozone therapy is described as non-traumatic, painless, and non-invasive, which enhances patient acceptance. The Cochrane Review identified multiple randomized controlled trials in dentistry that examined the impact of ozone therapy on both crown carious lesions and root lesions. It concluded that there is no evidence to suggest that the application of ozone alone arrests or reverses the decay process (Rius-Pérez et al. 2023). Additionally, another study showed that ozone and photodynamic therapy had a minimal effect on the lifetime of microorganisms from/within the cariogenic biofilm (Kuwano

et al. 2000; Wilczyńska-Borawska et al. 2011; Zhang and Ju 2012).

The dental pulp tissue in humans remains in a hypoxic environment, which could account for the prolonged survival of dental pulp cells following tooth extraction. The main core of the pulp is a cell-rich zone. This layer is formed by mesenchymal cells and star-shaped, spheroidal, and spindle-shaped fibroblasts. These cells interconnect to form a network (Boch et al. 2016; Grocholewicz et al. 2020). Both types of cells can divide and differentiate into fibroblasts under physiological or pathological stimuli or, through the transitional form known as pre-odontoblasts, into odontoblasts. Additionally, the pulp contains infiltrating cells involved in immune responses, including lymphocytes, granulocytes, plasma macrophages, mast cells, and antigen-presenting cells. Their numbers increase during inflammatory conditions of the tooth. The odontoblastic zone, situated in contact with dentine, contains odontoblasts, which are responsible for dentin production throughout their lifespan. Following damage, new odontoblasts may be formed from pulp fibroblasts or differentiated mesenchymal cells, forming so-called pseudo-odontoblasts (Rickard et al. 2004; Wong 2011; Pistritto et al. 2016). Beneath the odontoblasts lies a cell-free zone of Weil. Throughout the lifespan of a tooth, three types of dentine are formed. Primary dentin is generated by odontoblasts during the active secretory phase of tooth development. Once root formation is complete, odontoblasts secrete physiological secondary dentine. Under environmental stimuli, odontoblasts can generate tertiary dentine. However, more intense stimuli such as deep caries, cavity preparation, treatment, and restorative procedures may trigger apoptosis in odontoblasts.

Dental pulp cells, especially pulp stromal cells, due to their multipotentiality and regenerative ability, are regarded as a high-quality source in future regenerative therapies for a very diverse group of human diseases – from neurological and circulatory system diseases to diseases of the immune system and diabetes (Staniowski et al. 2021; Bryniarska-Kubiak et al. 2024). However, their acquisition and culture can pose many challenges. For our research purposes, we obtained cells from the permanent teeth of 30 healthy donors. However, only 20% of the cases yielded positive cell cultures. The remaining samples were contaminated or showed no signs of growth. Indeed, the challenges of hDPSC culture are well documented. Many authors draw attention to the risk of contamination of the obtained cells with oral bacterial flora, despite working in sterile conditions and the use of antibiotics to culture the isolated cells (Li et al. 2014; Andrukhov et al. 2019). Obtaining an appropriate number of living DPSCs from the dental pulp is another difficulty that appears despite using various methods to effectively break down the extracellular matrix (Lan et al. 2019). It is important to quickly process the extracted teeth, which affects their viability. It is also significant

that DPSCs undergo senescence with subsequent passages, which limits their use and expansion. Furthermore, DPSCs may have difficulty adhering to the culture surface or proliferating, and the use of coated culture dishes (e.g., with fibronectin, collagen, or poly-L-lysine) effectively enhances cell attachment (Nutti et al. 2016; Yamada et al. 2019).

Apoptosis, or programmed cell death, is precisely regulated at the gene level, resulting in the orderly and efficient removal of damaged cells, such as those experiencing DNA damage or during developmental processes. The process of apoptosis is intricate and entails various signaling pathways. Apoptosis can be induced in the cell through extrinsic or intrinsic pathways (Melet et al. 2008).

The intrinsic apoptotic pathway (mitochondria-dependent) is mediated by intracellular signals that converge at the mitochondrial level in response to various stress conditions. Irreversible genetic damage, hypoxia, extremely high concentrations of cytosolic Ca^{2+} , and strong oxidative stress are some of the factors that trigger the initiation of the intrinsic mitochondrial pathway. This leads to the activation of pro-apoptotic BH3-only members of the Bcl-2 family (Bax, Bak). Consequently, the anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1 are neutralized, resulting in the disruption of mitochondrial outer membrane permeability. This disruption causes proteins normally confined to the intermembrane space, such as cytochrome-c, to be released into the cytosol. Then, these apoptogenic factors, including cytochrome-c, play a crucial role in activating mitochondria-dependent apoptosis (Nagayoshi et al. 2004). Apoptosis in odontoblasts has been observed in rodent incisors and human molars at different ages. In cases where teeth are subject to severe stress, odontoblasts adjacent to the affected area may undergo apoptosis and subsequently be replaced by pulp progenitors-derived odontoblastic-like cells, leading to the formation of reparative dentine.

The balance between *Bax* and *Bcl-2* expression is critical. After ozonation, while there was an initial increase in *Bax* expression observed, a subsequent increase in *Bcl-2* would help to protect the cells and promote recovery. The ratio of *Bax* to *Bcl-2* determines the overall outcome in terms of cell survival or apoptosis. In the study presented, where ozone application was a stress factor for the dental pulp tissue, it was found that the basal level of apoptosis, as indicated by *Bax* expression, was higher compared to after the final ozone application. These findings confirm that pathological apoptosis induced by external stimuli, like ozone, can be detrimental to the dental pulp cells if excessive apoptosis is not prevented by overexpression (Müller et al. 2007; Naik et al. 2016; Benicio et al. 2018). Among the many regulators of apoptosis, Bcl-2, an anti-apoptotic protein, is expressed at different stages of tooth development. Research indicates that Bcl-2 expression is notably elevated in odontoblasts located beneath cavity preparations.

In our study, we found that *Bcl-2* gene expression was higher in pulp cells culture after 12 s of ozone application compared to after 6 s. These observations suggest that the regulation of apoptosis by *Bcl-2* may contribute to pulp homeostasis and damage-related remodeling to handle temporary injury caused by ozone exposure. However, how *Bcl-2* gene expression level is related to these processes is not clear in the present study, however, it appears that genetic manipulation of *Bcl-2* is a novel strategy to protect the tooth tissues under traumatic stress during different dental procedures (Akdeniz et al. 2018).

The results of conducted studies suggest that preconditioning with ROS (i.e., O_3 , H_2O_2) significantly induces overexpression of *Bcl-2* gene thereby protecting cells against apoptosis induced by oxidative stress. It has been demonstrated that ROS, generated within cells as a result of oxidative stress, acts as secondary signal transducers. A high concentration of ROS generates signals for transcription factors activating gene expression (Wang et al. 2014). Additionally, biomolecules modified by oxygen species may also serve as transmitters of extracellular signals (Yang et al. 2020). However, the protective effect of preconditioning is strictly dose-dependent (Jeon et al. 2022).

Ozonation is widely recognized for its ability to induce oxidative stress in various cell types, including lymphocytes. Excessive oxidative stress can lead to apoptosis of lymphocytes, potentially affecting their overall population in the sample. Our study was designed to investigate the effect of ozonation on specific immune markers present in lymphocytes. Lymphocytes from dental pulp were subjected to ozonation for 12 s. When comparing these cells to a control group, our findings showed no significant changes in the frequencies of Th cells, Tc cells, and B cells. This suggests that short-term ozonation does not drastically alter the major lymphocyte subpopulations. However, it is noteworthy that there was a slight decrease in the average frequency of B cells and Th cells in ozonated samples compared to controls. While this decrease was not statistically significant, it might indicate a subtle effect of ozonation that warrants further investigation, particularly with different exposure durations or higher doses. PD-1 and PD-L1 are critical immune checkpoint molecules involved in regulating immune responses (Müller et al. 2013). Considering their roles, we hypothesized that ozonation might influence their expression on lymphocytes. However, statistical analysis of our data showed that, despite some minor variations, ozonation did not significantly affect the expression levels of PD-1 and PD-L1 on B or T lymphocytes. This finding is significant because it suggests that short-term ozonation does not enhance or suppress immune checkpoint pathways mediated by PD-1/PD-L1. As a result, the functional status of these lymphocytes, in terms of activation and exhaustion, likely remains stable despite the oxidative stress induced by ozonation.

The interaction between CD200 and CD200R generally leads to the suppression of immune responses, creating an immunoregulatory environment. Changes in the expression of these molecules induced by oxidative stress may potentially enhance immune suppression. However, our experimental data showed that ozonation-induced oxidative stress had no effect on the expression levels of CD200 and CD200R on B lymphocytes. This indicates that the signaling pathways regulating these molecules resist oxidative challenges. The stability of PD-1, PD-L1, CD200, and CD200R expression under oxidative stress may indicate that the immune regulatory mechanisms involving these molecules are robust. Despite the oxidative challenges posed by ozonation, these pathways maintain their functional integrity. This immunity is critical to maintaining immune homeostasis and preventing excessive immune system suppression or activation.

5. Conclusions

Considering the potential of dental pulp cells in carious process and tissue regeneration, it is necessary to deepen scientific understanding of their behavior. Therefore, we conducted this research to investigate the possible effect of ozone on dental pulp cells.

In line with the broader literature, our results contribute to a better understanding of how oxidative therapies, such as ozone treatment, can modulate cellular and immune processes and highlight that oxidative treatment can influence cellular apoptosis pathways and immune response (Bryniarska et al. 2019).

Our study demonstrated that after ozone application *Bcl-2* promotes dental pulp cells' repair by preventing excessive apoptosis induced by ozone, evidenced by *Bax* gene expression. Additionally, *Bcl-2* gene expression was found higher in pulp cells after 12 s of ozone application compared to 6 s exposure.

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Furthermore, our study also indicates that short-term ozonation does not significantly impact the frequencies of major lymphocyte subpopulations or the expression of key immune regulatory molecules such as PD-1, PD-L1, CD200, and CD200R. These findings suggest that ozonation does not compromise lymphocyte function or the immune regulatory environment under the tested conditions. Future studies could explore the effects by changing the doses and durations of ozonation and its impact on lymphocytes under different physiological and pathological conditions, to fully elucidate the potential implications of ozone therapy on immune function.

Author Contributions

Conceptualization, M.O. and R.CH.; methodology, J.K. and K.G.; validation, J.K., M.O. and R.CH.; formal analysis, M.P., A.M.; investigation, M.O., J.K.; resources, R.CH.; data curation, RCH; writing—original draft preparation, M.O., J.K., R.CH., M.P., A.M., P.S.; writing—review and editing, R.CH.; visualization, M.O., M.P., A.M.; supervision, R.CH.; project administration, M.P; funding acquisition, R.CH., J.K.

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Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest

The authors declare no conflict of interest.

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