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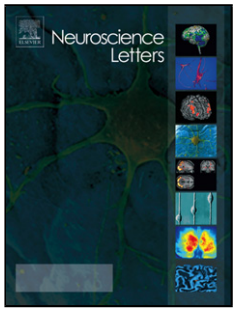

Ozone influences migration and proliferation of neural stem cells *in vitro*

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PII: S0304-3940(20)30660-1

DOI: https://doi.org/10.1016/j.neulet.2020.135390

Reference: NSL 135390

To appear in: *Neuroscience Letters*

Received Date: 22 April 2020

Revised Date: 9 September 2020

Accepted Date: 11 September 2020

Please cite this article as: Tricarico G, Isakovic J, Song MS, Rustichelli F, Travagli V, Mitrecic D, Ozone influences migration and proliferation of neural stem cells *in vitro*, *Neuroscience Letters* (2020), doi: https://doi.org/10.1016/j.neulet.2020.135390

Ozone influences migration and proliferation of neural stem cells *in vitro*

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Highlights

- Ozone increases neural stem cell migration and cytokine release
- To a smaller extent, ozone also increases neural stem cell proliferation
- Due to its electrical properties, ozone has a potential use in neurorestoration

Abstract

Ozone (O_3) is a short-lived molecule which can be produced in a controlled reaction when oxygen is exposed to electric discharge. In the last few decades, many publications dealing both with animals and humans reported beneficial effects of ozone administration linked to its immunomodulatory and protective role against cellular damage. This is the first work which brings insight into how ozone influences cells of neural lineage *in vitro* and hypothesizes the potential molecular and novel electromagnetic mechanisms behind its action. By using neural stem cells, we show that ozone, especially in concentrations of around 11 µg/ml, significantly increases the speed of neural cell migration. With much lower effects, it also increases cell proliferation and cytokine production. Results of this study, at least partly, explain the observed beneficial effects of ozone in diseases of the nervous system tested on animal models and in human clinical trials. Therefore, here described effects of ozone on cellular level represent a firm basis for further investigation of possible applications of ozone in regeneration of the nervous system.

Keywords: ozone, neural stem cells, migration, proliferation, nervous system

Introduction

Ozone (O_3) is a highly reactive inorganic molecule, as well as one of the most powerful oxidising agents. With three atoms of oxygen, ozone has the ability to oxidise the phospholipids and lipoproteins in the cell's membrane, impacting its integrity and causing an increase in cytokine release.

Since the mechanism which influences membrane phospholipids interferes with cell signalling, many experiments tested effects of administration of ozone on cells and tissues. Recent publications reported positive effect of ozone in several pathological conditions. Ozone is already well established in treatment of oral cavity, where it improves healing and decreases pain [1]. This approach has been expanded to wound healing in skin, where the mechanism of healing has been attributed to increased speed of cell migration [2].

As it has recently also been shown that ozone influences the cell's immune response and levels of chronic oxidative stress [3–6], it was plausible to hypothesize that ozone might also have a beneficial influence on the nervous tissue as well. And indeed, the most recent publication revealed positive results in improving cognitive status of mice affected by Alzheimer's disease, which was, at least partially, explained by reduced accumulation of amyloid precursor protein (APP) in the brain of animals treated by ozone [7]. Equally, ozone appears to bring improvements in both animal models [8] and patients with multiple sclerosis [9].

In this work we aimed to get insight into the cellular mechanism of influence of ozone on cells of the nervous tissue. Because brain diseases affect more than 30% of people worldwide, with their chronicity and limited therapeutic options, they represent one of the highest burdens on the modern society. Apart from pharmacological approaches, which often lead to large disappointment due to high cost of investment in new drugs and a very high rate of failures, other approaches are urgently needed [10].

Stem cells represent one of the most promising tools of the modern medicine. They became an almost obligatory part in neuroscience research in the past decade [11] and they offer new possibilities in modern therapeutic applications [12,13].

Due to the fact that differentiation and migration of neuroectodermal cells are the backbone of many pathological conditions, and since some publications reported positive effect of ozone on conditions affecting nervous tissue, in this work we tested the influence of ozone on neuroectodermal cells - neuronal precursors and differentiated neurons. We quantified their proliferation, migration, cytokine release and cell survival, as well as suggested potential molecular and novel electromagnetic mechanisms behind its action. With that, this work clearly shows that ozone positively influences neural stem cells, which at least partly explains observed beneficial effects of ozone administration in vivo and is an important starting point for future research on stem cell therapy optimisation.

Materials and Methods:

To obtain neuronal cells we have isolated neural stem cells from mouse embryos. For this specific purpose we used the telencephalic wall of 14-day-old embryos. This

allowed us to obtain uniform population of precursors of all cells in the nervous system, including neurons, neural crest cells and glia. Cells were cultivated in DMEM/F-12, B-27, N-2, Pen Strep (Penicillin-Streptomycin) with added FGF-β (basic fibroblast growth factor) and EGF (epidermal growth factor) growth factors.

After cultivation for 2 weeks, which included 4 passages, neural stem cells have been seeded in a 24-well plate which has previously been treated with poly-D-lysine and laminin to mimic their natural environment and help them attach to the surface. To confirm that we used a homogeneous population of neural stem cells, we performed immunocytochemistry against nestin, a marker protein for neural stem cells, which was then shown to be expressed in 95% of analyzed cells (Fig. 1).

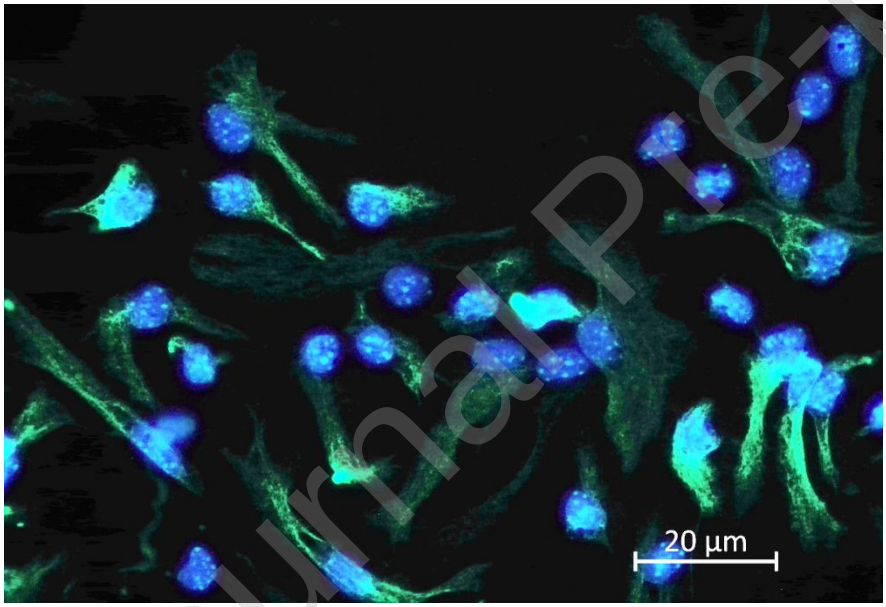


Figure 1. Flourescence immunocytochemistry for nestin (green signal) on NSC cultures. Over 95% of analysed cells express nestin. Blue signal corresponds to cell nuclei.

Moreover, differentiation essay using neural stem cells seeded to poly-D-lysine surface in the neural medium without growth factors revealed that after 5 days, at least 50% of cells express markers of neural lineages, Map2 and Tuj1.

For the purpose of quantifying the effect of various ozone concentrations on neuroectodermal cells, we applied an instrument (MIDI Ozon Active – Medica Srl Bologna) which transforms oxygen to ozone (3 molecules of oxygen to 2 molecules of ozone via process of electrical charging), enabling us to produce different mixtures of oxygen and ozone, depending on the desired concentrations.

In order to perform the experiment, we used the first two and the last two wells as untreated controls (CTRL 1, CTRL 2) and treated the following 14 wells with 14 different total amounts of ozone in an oxygen-ozone mixture at different levels of concentration: 5 µg/ml , 7 µg/ml, 9 µg/ml, 11 µg/ml, 13µg/ml, 15 µg/ml, 17 µg/ml, 19 µg/ml, 21 µg/ml, 25 µg/ml, 29 µg/ml, 33 µg/ml, 40 µg/ml, 50 µg/ml. Any treatment was made by inoculation, with a needle mounted on a syringe, of 2.5ml of oxygen-ozone mixture prepared by an ozone-oxygen production device MIDI Ozon Active (Medica Srl).

Cells were cultivated for 3 days in 5% CO_2 and were scanned by EVOS Life Sciences Automated System for 2 hours on each of the days, in a way that every 2 minutes photo of one region within the well has been taken.

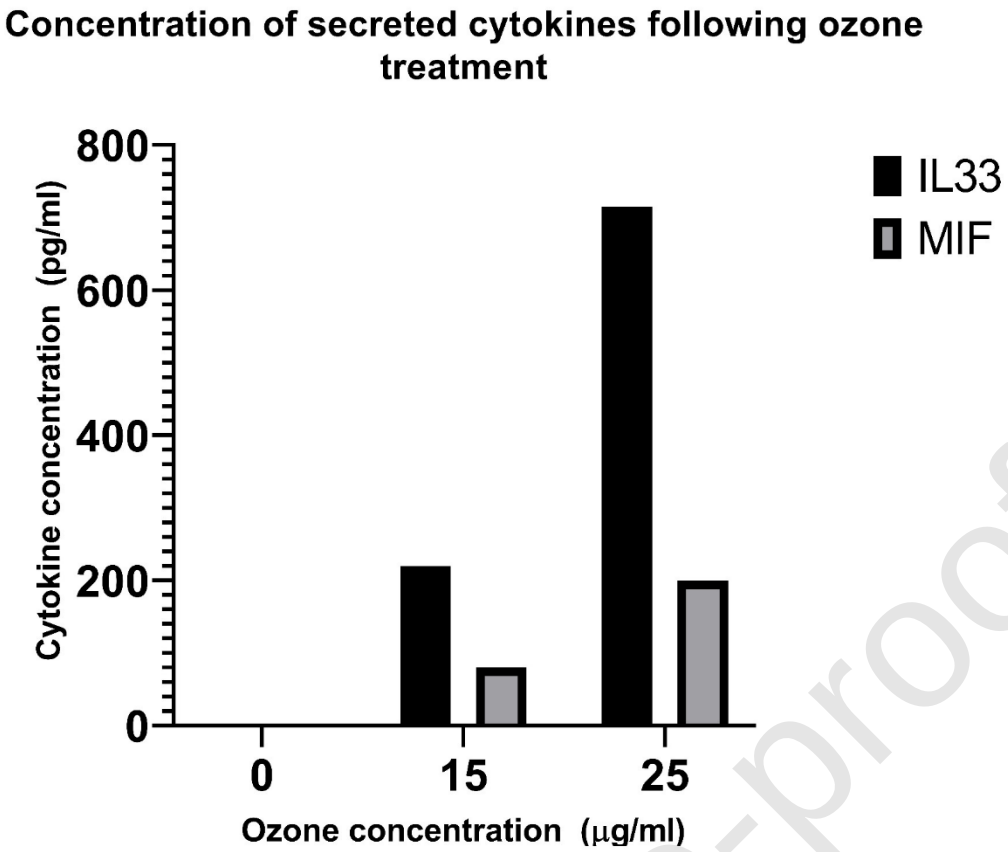
Then, using cell tracking software by EVOS Thermo Fisher and Image J, average and maximal speed of migration and proliferation were analyzed and compared in time and in between administered ozone concentrations.

In order to measure the concentration of secreted cytokines interleukin 33 (IL 33) as well as macrophage migration inhibitory factor (MIF), which are known to be produced by neurons, and demonstrate the machine's ability to produce ozone, mediums in which cells were grown (CTRL 1, 15 µg/ml and 25 µg/ml groups) were centrifuged at 1,500 rpm for 10 min at 4°C. Supernatant was stored at -80°C and official kit instructions were followed (IL33, BMS6025, ThermoFisher Scientific; MIF, ab209885, Abcam). After obtaining a standard curve, concentrations were measured on the microplate absorbance reader for the three respective groups: control, 15 and 25 µg/ml of ozone (iMark, Bio Rad).

Results

In order to check whether ozone is being produced by the machine, a positive control experiment was done, e.g., we measured IL33 and MIF cytokine concentration in response to ozone treatment (Fig. 2).

As our results show, cytokine release drastically increased upon ozone application, suggesting that, as expected, ozone has a strong impact on cytokine production (Fig. 2).



OZONE CONCENTRATION	CYTOKINE CONCENTRATION	
	IL33	MIF
0 µg/ml	0	0
15 µg/ml	220 pg/ml	80 pg/ml
25 µg/ml	715 pg/ml	200 pg/ml

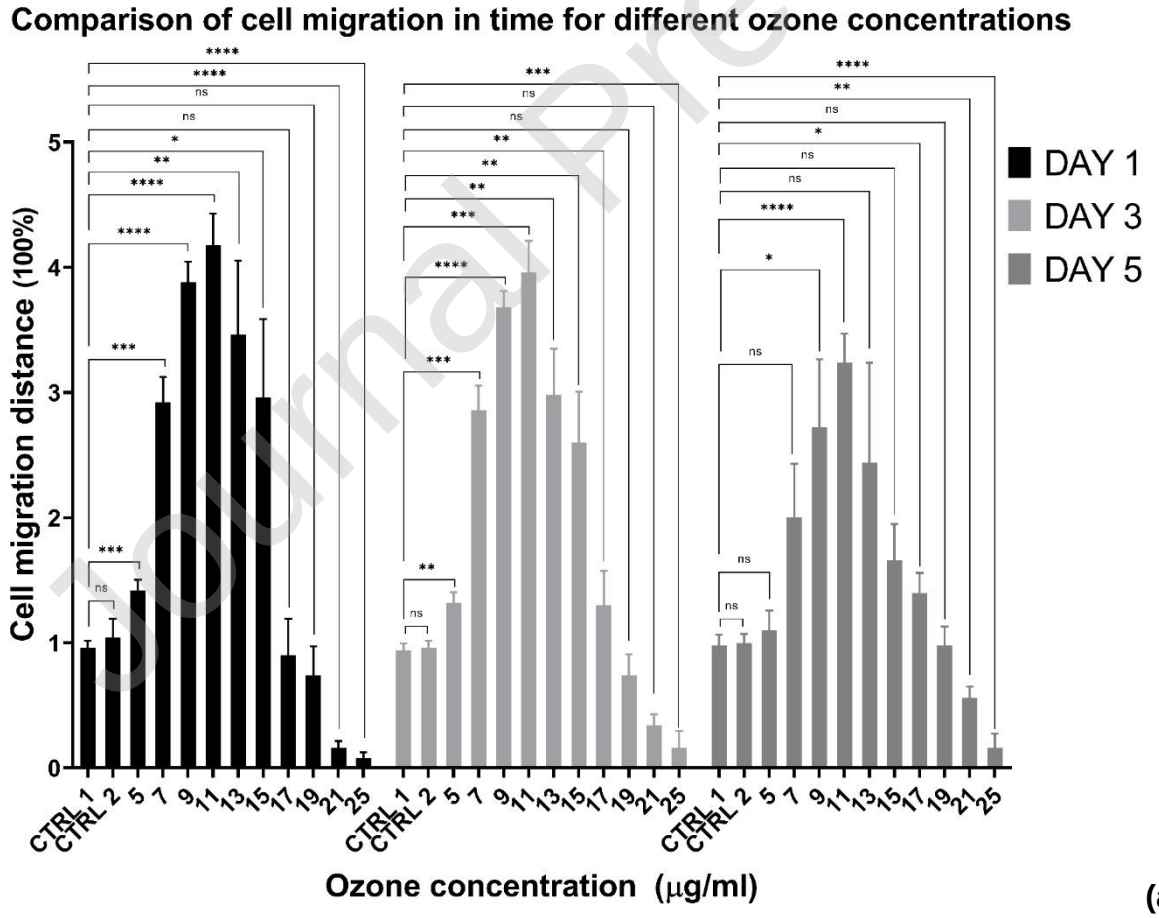
Figure 2. Concentration of cytokines secreted by NSC following treatment with 15 and 25 µg/ml of ozone and a control group with no ozone application. This graph includes measurements of IL33 and MIF cytokine release after 0, 15 and 25 µg/ml of ozone application. Upon increase of ozone concentration, the cells exhibited a surge in cytokine release for both concentrations.

Whilst the concentration of IL33 rose from 220 pg/ml (at 15 µg/ml of ozone) to 715 pg/ml (at 25 µg/ml of ozone), the increase in MIF was not so drastic, going from 80 pg/ml (at 15 µg/ml of ozone) to 200 pg/ml (at 25 µg/ml of ozone) (Fig.2) – nevertheless demonstrating the efficiency of the ozone-producing device.

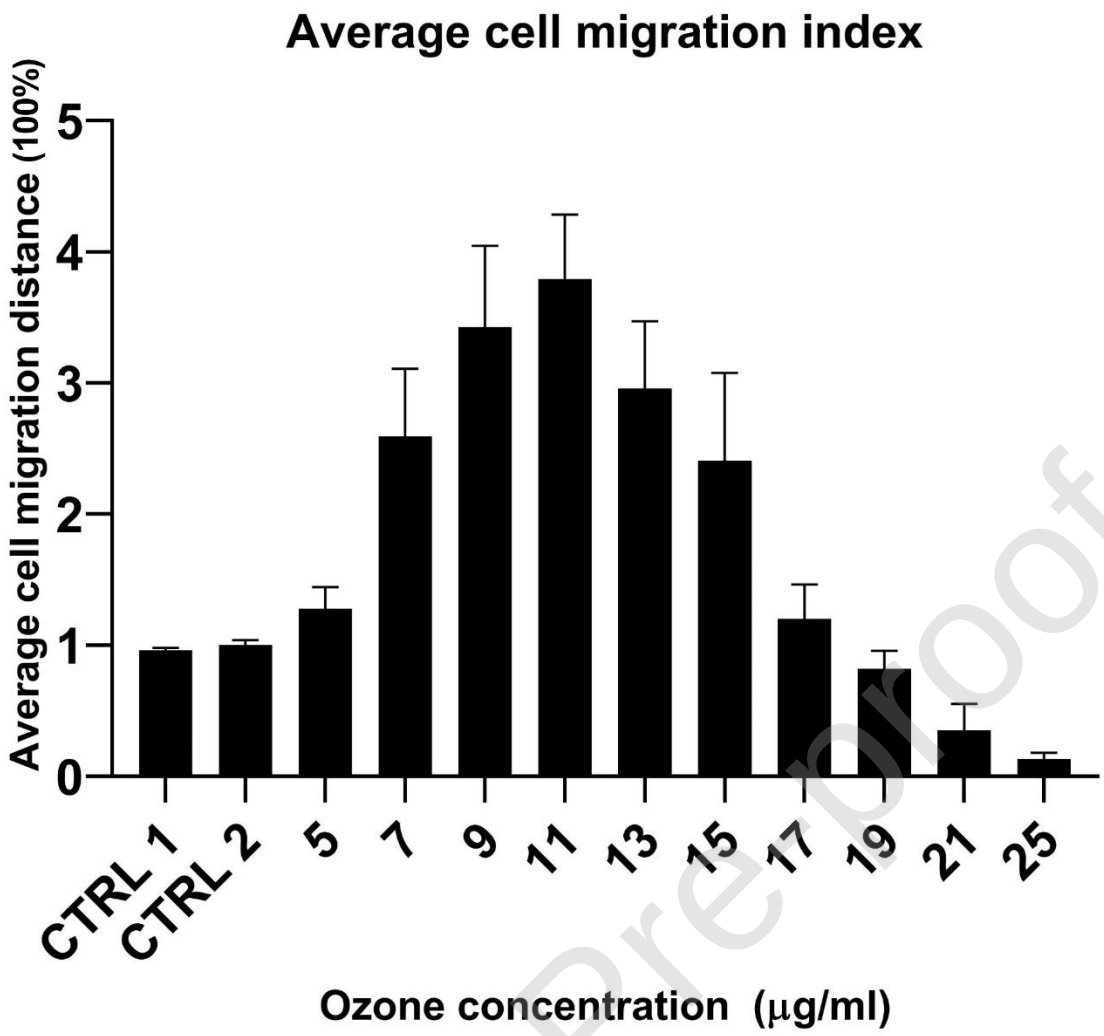
Next, to quantify the influence on ozone on neuroectodermal cells, we measured cell migration and proliferation. Statistical analysis of the obtained data was done using two-way repeated measures ANOVA in GraphPad Prism statistical software.

We first measured the distance of migration by a cell tracking software, Cell Tracker. By comparing this value with the initial point, we obtained a percentage difference in cell migration.

Analyses of average migration on days 1, 3 and 5 after treatment with different concentration of ozone revealed the migration index for each of the 5 repeated measurements in the observed days which can be seen in Fig. 3a.



(a)



(b)

Fig. 3 a Migration index for various concentrations on day 1 (dark gray), 3 (light gray) and 5 (medium gray) of ozone administration. The graph includes averages of values obtained with 5 repeated measurements for each concentration within one well and standard deviation corresponding to each measurement. ***b*** A graph of the average cell migration as averaged through three days for each of the corresponding concentration, including the standard deviation for each concentration (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

As it is seen in Fig. 3a, the average migration index can be approximated to be following a Gaussian distribution for each of the corresponding days. Within the first

day, a statistically significant difference in cell migration can be observed for almost all ozone concentrations (5 – 15 µg/ml and 21 – 25 µg/ml) except for 17 and 19 µg/ml. The largest effect can be seen for concentrations 9, 11, 21 and 25 µg/ml, for which $p < 0.0001$. Whilst groups 9 and 11 µg/ml exhibit a significant increase, cells in groups 21 and 25 µg/ml showcase a drastic decrease in migration, when compared to the control group.

A similar pattern can also be observed for day 3, where the largest statistical significances are observed for groups 7, 9, 11 and 25 µg/ml. Whilst only group 9 µg/ml appears to have $p < 0.0001$, other three groups have $p < 0.001$, still making the effect in increased or decreased migration extremely statistically significant.

Extreme statistical significance ($p < 0.001$) on day 5 can only be observed between groups 11 and 25 µg/ml as compared to the control. This suggests that the migration of NSC treated with the aforementioned ozone concentrations stayed most consistent throughout days and exhibits little changes as those two concentrations also demonstrated extreme statistical significance from the control group during days 1 and 3.

On the other hand, cells in groups 5, 7, 9, 15 and 21 µg/ml exhibit most changes throughout the days. Whilst the cells treated with 5 µg/ml went from $p = 0.0005$ (***) at day 1, to $p = 0.0016$ (**) at day 3 and $p = 0.8971$ (ns) at day 5, those treated with 7 µg/ml had $p = 0.0001$ (***) at days 1 and 3 and $p = 0.0638$ (ns) at day 5. Following this, the 9 µg/ml group had $p < 0.0001$ (****) at days 1 and 3 and only $p = 0.0224$ (*) at day 5, suggesting a large decrease in the effect of ozone on cell migration. Next,

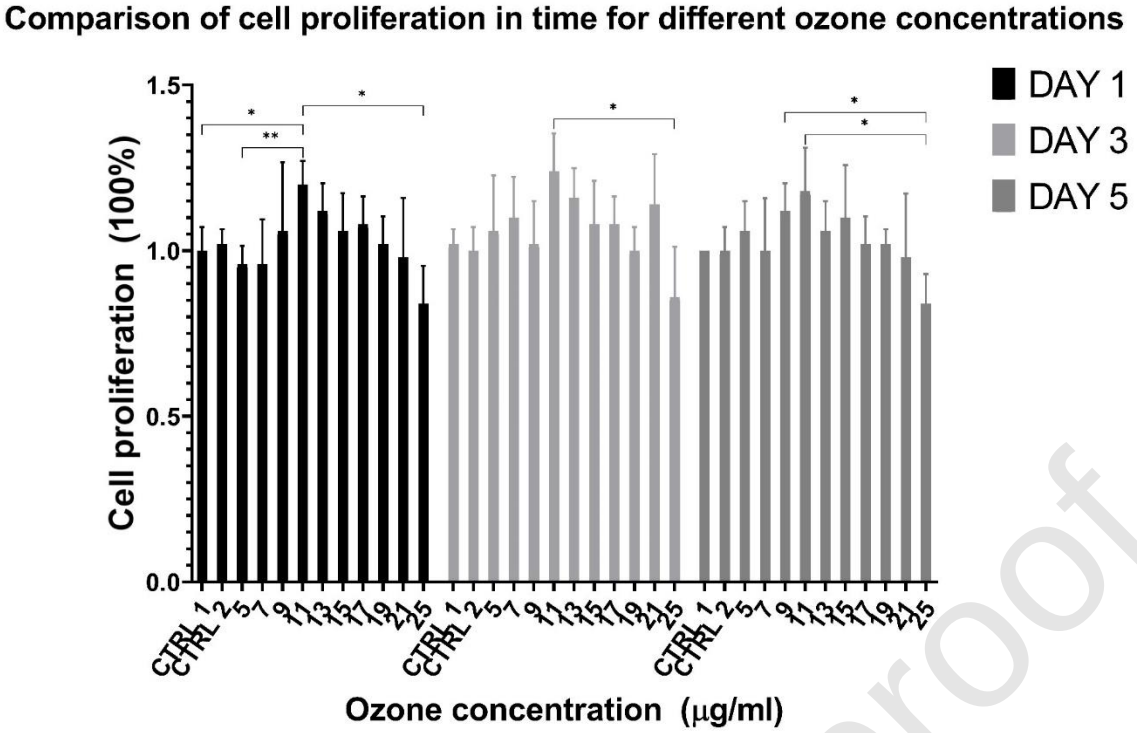
cells treated by 15 µg/ml of ozone had $p = 0.0236$ (*) at day 1, the effect increased at day 3, leaving them at $p = 0.0089$ (**), and decreased again for day 5 to $p = 0.0612$ (ns). Finally, as much as those cells in group 21 µg/ml showed a decrease in migration for day 1, with $p < 0.0001$ (****), the effect diminished on day 3, with $p = 0.0001$ (***), and day 5, with $p = 0.0016$ (**), implying a generalized recovery of cell migration after 5 days.

Moreover, even though we used concentrations above 25 µg/ml, majority of the cells in wells with 33, 40 and 50 µg/ml of ozone have died, so no measurements could be done on them.

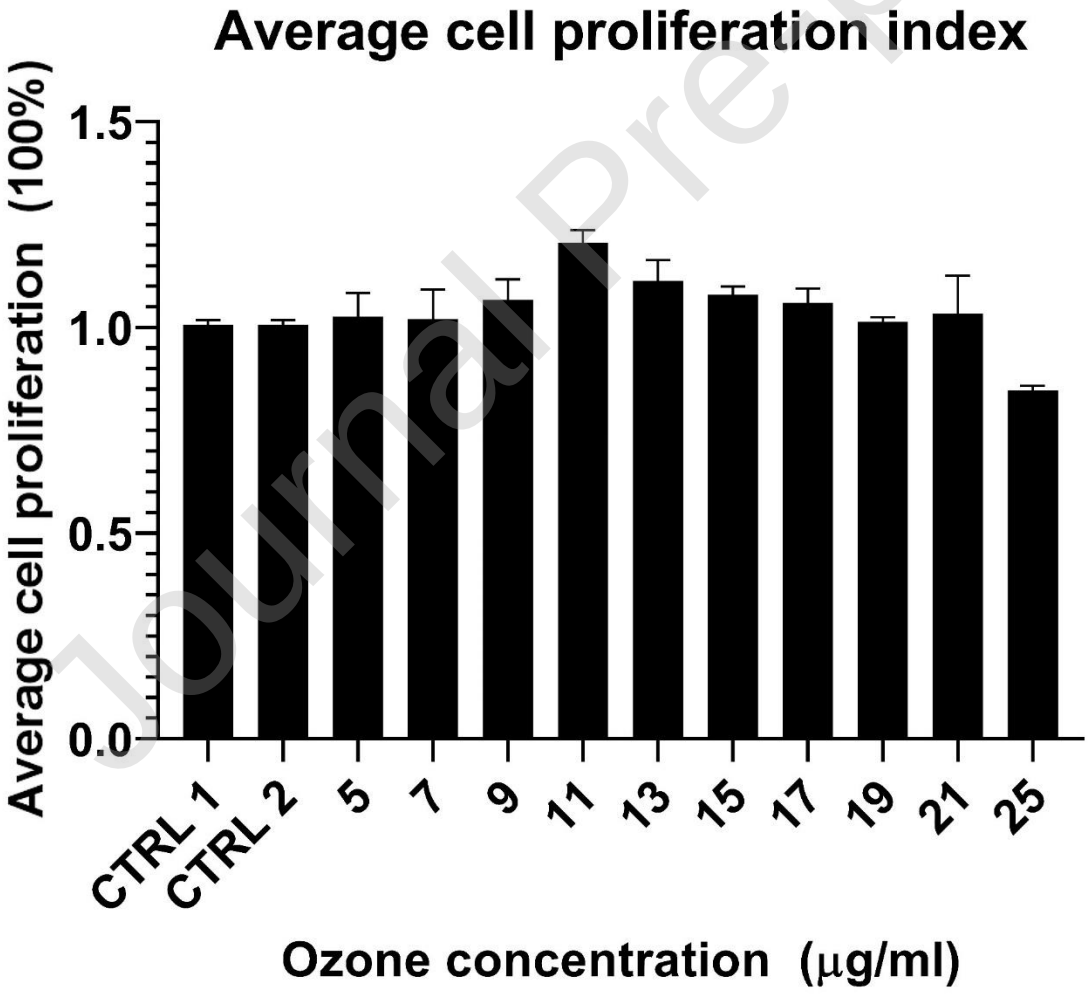
In order to observe a general data trend, average migration index analysis has been done by averaging the migration for each concentration throughout the days and represented those values with corresponding standard deviations, Fig. 3b.

What appears striking in Fig. 3b is the fact that all the groups, except the two control groups, groups 13 and 15 µg/ml and 9 and 11 µg/ml amongst one another, exhibit a significant difference between them. Ozone concentrations from 5 to 11 µg/ml caused a general increase in cell migration whilst those from 13 to 25 µg/ml induced a decrease in cell migration.

In order to quantify cell proliferation, we counted the number of cells in each well, compared it to the initial number of cells and obtained a number quantifying total cell proliferation, in the form of a percentage compared to the initial number.


Comparison of cell proliferation in time for different ozone concentrations
DAY 1
DAY 3
DAY 5
Cell proliferation (100%)
1.5
1.0
0.5
0.0
*
**
*
*
*
*
CTRL 1
CTRL 2
5
7
9
11
13
15
17
19
21
25
Ozone concentration (μg/ml)

(a)


Average cell proliferation index
Average cell proliferation (100%)
1.5
1.0
0.5
0.0
CTRL 1
CTRL 2
5
7
9
11
13
15
17
19
21
25
Ozone concentration (μg/ml)

(b)

Fig. 4 a Proliferation index for various concentrations on day 1 (dark gray), 3 (light gray) and 5 (medium gray) of ozone administration. The graph includes averages of values obtained with 5 repeated measurements for each concentration within one well and standard deviation corresponding to each measurement. **b** Comparison of the average proliferation-migration index for each day of ozone administration and corresponding ozone concentration. This graph also includes standard deviation for each computed average over 5 measurements per day and concentration. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

As opposed to an obvious statistical significance that was seen in cell migration during different time points, no statistical significance in cell proliferation in different time points in can be seen Fig. 4a.

Finally, whilst comparing the average cell proliferation between different ozone concentrations, as depicted in Fig. 4b, we can observe that average cell proliferation peaks at 11 µg/ml for all three time points (day 1, day 3 and day 5). There also exists a statistical significance in cell proliferation between 11 µg/ml group and groups 25 µg/ml and the control ($p < 0.05$) as well as group 5 µg/ml ($p < 0.01$) for day 1, and groups 11 and 25 µg/ml for both days 3 and 5 ($p < 0.05$). Last statistical significance can be observed between groups 9 and 25 µg/ml with $p < 0.05$.

All other cells treated with different ozone concentrations exhibit no statistical significance amongst each other or as compared to the control group (Fig. 4b).

From this it can be concluded that ozone at proper concentration is able to stimulate neural stem cells for faster migration and relatively increased proliferation.

Discussion

In this work we have shown that ozone influences neural stem cells *in vitro*. Depending on the concentration, ozone significantly influences cytokine release, distance of cell migration and causes slight changes in cell proliferation (Fig 2., Fig.3 and Fig.4).

When it comes to cytokine release, this experiment has demonstrated changes in IL33, which rose from 220 pg/ml (at 15 µg/ml of ozone) to 715 pg/ml (at 25 µg/ml of ozone), as well as an increase in MIF, going from 80 pg/ml (at 15 µg/ml of ozone) to 200 pg/ml (at 25 µg/ml of ozone) (Fig.2). Coupled with current research in the field [14,15], this finding suggests the idea that ozone can greatly influence pro-inflammatory cytokine production and influence inflammation. This can have both beneficial and harmful effects, depending on ozone concentration and corresponding cytokine production.

Next, our study has shown that varied concentrations of ozone impact, to a large extent, the distance of cell migration (Fig. 3). Interestingly, almost all groups (except for group 19 µg/ml) have shown some significant difference in cell migration distance in time, on days 1, 3 and 5 post-ozone application (Fig. 3a). When observing the average migration distance for each ozone concentration, with the value averaged over the three time points, there exists a significant difference not only between the experimental and control groups but also in between groups themselves (Fig. 3b).

Most interestingly, there is a large difference between average cell migration distance in groups 5 vs. 7 µg/ml, 7 vs. 9 µg/ml, 15 vs. 17 µg/ml and 19 vs. 21 and 25 µg/ml (Fig. 3b). Observing the shape of the histogram, ozone appeared to be having two-fold influence on NSC migration. It increased the average migration distance for groups 5 – 15 µg/ml, as compared to the control, therefore having a beneficial effect and decreased the average migration distance for groups 17, 21 and 25 µg/ml, where it had a detrimental effect. This is supported by the fact that cells in groups 17, 21 and 25 µg/ml, exhibited significant decrease in cell migration as compared to the control groups (Fig. 3a). No difference in cell migration was observed in group 19 µg/ml, as compared to the control. Since Fig. 3b. also shows that average NSC migration distance in groups 9 and 11 µg/ml reached its peak, this suggests the existence of an optimal ozone concentration for increasing the migration distance of NSCs.

On the other hand, ozone has also, but to a much smaller extent, increased average cell proliferation (Fig. 4). Observing Fig. 4a, there appears to be no significant difference between cell proliferation during three different time points (1-, 3- and 5-days post-ozone application), suggesting that, as opposed to cell migration, ozone does not appear to be altering cellular proliferation dynamics in time, at least not to an observable extent. Although, slight differences were seen when comparing the average cell proliferation for different ozone concentrations where an increase in average cell proliferation occurred from the control to the 11 µg/ml group ($p < 0.05$) as well from 5 to 11 µg/ml ($p < 0.01$) (Fig. 4b). No significant difference for average cell proliferation was observed between the control and 5 - 9 µg/ml as well as 13 – 21 µg/ml groups (Fig. 4b).

Apart from a well proven positive effects of ozone in wound healing [16] and in dentistry [17], a growing list of evidence report benefits of using ozone in other organ systems as well [18,19]. Among these, we were intrigued by reports describing positive effects of ozone in treating the brain, in both animal [7,8,20] and human models [9,21]. At the same time, basic experiments showing direct effects of ozone on cells of neural lineage were lacking. With that, this is the very first study which shows how ozone influences cells of neuroectodermal origin and aims to put forth hypothetical molecular and electromagnetic mechanisms behind ozone's action on neural stem cells.

On the other hand, when thinking of common mechanisms which might be involved in the observed influence of ozone, there is, indeed, some work performed on other cell types, allowing us to draw parallels between these studies and suggest its importance in future stem cell research. Constanzo et al. have shown that ozone activates the cell anti-oxidative response pathways in human adipose derived adult stem cells (hADAS) and promotes lipid accumulation, all without causing any deleterious effects [22]. Whilst high levels of ozone or pure oxygen application increase the cell death by induction of many reactive oxygen species (ROS), they suggested that controlled concentrations of ozone promote positive anti-stress response, positively impacting cell proliferation, survival and differentiation [22].

When analysing the molecular pathways potentially involved in the phenomena we observed, one of the crucial molecular targets of ozone reported in the literature is nuclear factor-erythroid 2-related factor 2 (Nrf2) [23]. It has been shown that ozone increases nuclear translocation of Nrf2 and induces a dose-dependent increase in Antioxidant Responsive Element (ARE)-driven gene transcription, maintaining redox

homeostasis [24]. This mechanism, by which cells fight against oxidative stress resulting from mild ozonisation, prevents DNA damage, enhances mitochondrial function and suppresses inflammation [24].

On top of this, Nrf2 is also a transcription factor which plays a role in maintaining proper proliferation and differentiation rates of NSCs [25]. In this way Nrf2 makes a very interesting link between antioxidant roles (for example modulated over SOD1) and those ones linked to cell survival, growth, differentiation and proliferation [25]. Since Nrf2 has recently been clearly recognized as a protective factor for various brain diseases, increase of its activity upon ozone stimulation might explain at least part of our observed phenomena [26].

Other molecular mechanisms most likely linked to observed phenomena are Heme Oxygenase-1 (HO-1) and tumour necrosis factor α (TNF-α) modulation. As HO-1 is a Nrf2 target gene with a protective function, when it comes to the role of HO-1 in NSC functioning, Bonnamain et al. have shown that HO-1 is one of the key factors involved in mediation of NSC immunosuppressive activity [27,28]. On top of that, ozone can also alleviate oxidative damage by increasing GSH-Px, SOD and CAT and decreasing mitochondrial damage [29].

Stepping away from molecular mechanisms of ozone action upon NSCs, another interesting aspect of this interaction is the electrical one. As ozone represents a point charge, necessarily having the nature of electric fields as other point charges, the effects of ozone on cells can be seen as similar to the ones exerted by electromagnetic fields [30,31]. With that, ozone has the ability to not only impact the molecular

pathways for pro- or anti-inflammatory response or disrupt the cell's cytoplasmic membrane integrity through oxidation of phospholipids and lipoproteins, but it also has the ability to modify the external surface charge distribution of cells, as well as their lipid structure organisation and ion channel distribution. This can lead to a change in the embedded membrane components kinetics as well as modification of the opening and closing of voltage gated channels. Thus, we believe that at least part of phenomena we report in this publication is linked to the change of external surface charge and its influence on cell interactions. One explanation for this could be the fact that ozone, with its innate electric field, causes an electric charge accumulation or an increased electrical or electrochemical gradient, therefore impacting also the rates and the distances of NSC migration.

Conclusion

Although more studies will be needed to get deeper insight in all effects of ozone on the nervous tissue, with a special focus on its electromagnetic properties, as well as to identify its main molecular targets, in this work we have clearly shown that majority of effects observed during ozonisation of other cell types are also present in those of neural lineages with some specific differences. Increased migration and proliferation, might, alongside proven protection against oxidative stress, serve as a specific mechanism by which ozone improves regeneration of the nervous tissue.

CRediT author statement:

Gerardo Tricarico: Conceptualization, Investigation, Resources, Writing – Original Draft, Funding acquisition. ***Jasmina Isakovic:*** Conceptualisation, Methodology,

Software, Formal analysis, Investigation, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualisation, Project administration. **Min Suk Song:** Software, Data curation. **Franco Rustichelli:** Writing – Original Draft, Writing – Review & Editing, Supervision. **Valter Travagli:** Validation, Writing – Review & Editing. **Dinko Mitrecic:** Conceptualisation, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Supervision, Project administration. Funding acquisition.

Acknowledgements:

We thank COST Action 16122, Biomaterials and advanced physical techniques for regenerative cardiology and neurology (BIONECA). D.M. is supported by the Croatian Science Foundation (IP-2016-06-9451) and co-financed by the Scientific Centre of Excellence for Basic, Clinical and Translational Neuroscience (project “Experimental and clinical research of hypoxic-ischemic damage in perinatal and adult brain”; GA KK01.1.1.01.0007 funded by the European Union through the European Regional Development Fund).

Author Contributions:

G.T., F.R., D.M. and J.I. conceived the experiments and wrote the manuscript. G.T., D.M. and J.I. performed the experiments. J.I., MS.S, and D.M. processed the experimental data. J.I. and D.M. designed the figures, performed the analysis and interpreted the results. G.T. and D.M. provided the materials and reagents. V.T.

provided expertise and feedback in manuscript review. F.R. and D.M. secured the funding.

Declaration of interests:

The device used to perform the experiments is MIDI Ozon Active, produced by Medica Srl. The authors of this paper are in no way affiliated with the device manufacturer or distributor and declare no personal interest, direct or indirect, in the subject matter and materials discussed in this manuscript.

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