

# Pre-application of pulsed magnetic field protects oxidative stress-induced apoptosis of vascular smooth muscle cells

F. Cicek\*, C. Coskun, I. Baldan, M. Tokuş, I. Gunay

Department of Biophysics, Faculty of Medicine, Cukurova University, Adana, Turkey

## ► Original article

### \*Corresponding author:

Figen Cicek, Ph.D.,

E-mail: fcicek@cu.edu.tr

Received: December 2020

Final revised: May 2021

Accepted: May 2021

Int. J. Radiat. Res., April 2022;  
20(2): 277-282

DOI: 10.52547/ijrr.20.2.4

**Keywords:** Pulsed magnetic field, oxidative stress, VSMC, apoptosis.

## ABSTRACT

**Background:** Pulsed magnetic field (PMF) application is an alternative therapy method used especially in the treatment of musculoskeletal system diseases. However, its effects on the vascular system are unclear. On the other hand, an imbalance in the synthesis of reactive oxygen species (ROS) can cause crucial vascular diseases such as hypertension or atherosclerosis. Therefore, this study aimed to elucidate the modulatory effect of PMF pre-exposure and the protective role of the application on ROS-related vascular dysfunctions. **Materials and Methods:** Rats were exposed to 1.5 mT, 40 Hz PMF for 30 days. Vascular smooth muscle cells (VSMCs) were isolated enzymatically at the end of the application. Cell proliferation in the presence or absence of hydrogen peroxide ( $H_2O_2$ , oxidative stress and apoptosis inducer) was determined by MTT assay. Caspase-3 activity and protein expressions were also determined. **Results:** Incubation of VSMCs with  $H_2O_2$  decreased the cell proliferation dose-dependently. However, the cells isolated from PMF pre-treated rats had higher proliferation levels at high dose  $H_2O_2$  (1 mM) than their controls. Moreover, PMF pre-applied cells had less caspase-3 expression at high dose  $H_2O_2$  incubation. A similar effect of PMF was also observed in caspase-3 enzyme activity. **Conclusion:** The present study demonstrated that PMF pre-exposed cells showed resistance to  $H_2O_2$ -induced oxidative stress. Notably, the decreased activity and expression of caspase-3 in PMF pre-treated groups indicated that PMF has regulatory effects on apoptosis formation mechanisms. The present study demonstrated that PMF pre-application should be considered as protective in the development of vascular diseases.

## INTRODUCTION

Reactive oxygen species (ROS) play an important role in the generation and progression of cardiovascular diseases with their numerous actions on cell metabolism<sup>(1,2)</sup>. Hydrogen peroxide ( $H_2O_2$ ) is a permanent cell membrane ROS molecule, produced by autocrine or paracrine signaling. The production and degradation of  $H_2O_2$  stay in balance throughout normal physiological processes<sup>(3,4)</sup>. In vascular smooth muscle cells (VSMCs), ROS production at high levels can impair cell survival and apoptosis mechanisms, leading to vascular disorders such as hypertension, atherosclerosis, and diabetes<sup>(2,5,6)</sup>. Therefore, treating or blocking of ROS-induced apoptotic damage may prevent vascular diseases.

It has been shown previously that magnetic field applications are effective in the treatment of tumor growth<sup>(7)</sup>. In monocytic cell lines, a pulsed magnetic field (PMF) increased the Bcl-2 anti-apoptotic protein expression via the caspase-independent pathway<sup>(8)</sup>. In another study, the application decreased apoptosis in the U397 human lymphoid cell line treated with puromycin, a telomerase and apoptosis inhibitor<sup>(9)</sup>. Experiments using nontumorigenic cells (MCF10

and human breast adenocarcinoma cells (MCF7) have also shown that PMF enhanced apoptosis in adenocarcinoma cells while it had no effect on nontumorigenic cells<sup>(10)</sup>. Furthermore, previous data have demonstrated that in vivo PMF application inhibits tumor cell growth via inducing apoptosis and blocking neovascularisation formation in tumor development<sup>(11)</sup>.

The protective effect of PMF on oxidative stress was also shown in previous studies<sup>(12)</sup>. PMF application at 2.5 mT and 50 Hz reduced oxidative stress following spinal cord injury<sup>(13)</sup>. The cytoprotective action of PMF pre-application was also demonstrated in  $H_2O_2$  treated human neuroblastoma (SH-SY5Y) cell lines with reduced ROS production<sup>(14)</sup>. Although these studies provided evidence for the preventive effects of PMF at apoptosis, its action on VSMCs remains unclear. Thus, this study aimed to observe the protective effect of in vivo PMF application on the vascular system at the cellular level, for the first time.

Since some cardiovascular diseases are related to an imbalance of apoptosis by ROS, we set out to evaluate the cytoprotective effect of PMF through ROS-induced apoptosis at vascular cells.

## MATERIALS AND METHODS

### Animals

The study and all the animal procedures were approved and performed in accordance with the Cukurova University Local Ethics Committee on Animal Experiments standards (approval no: 31.01.2017/1)

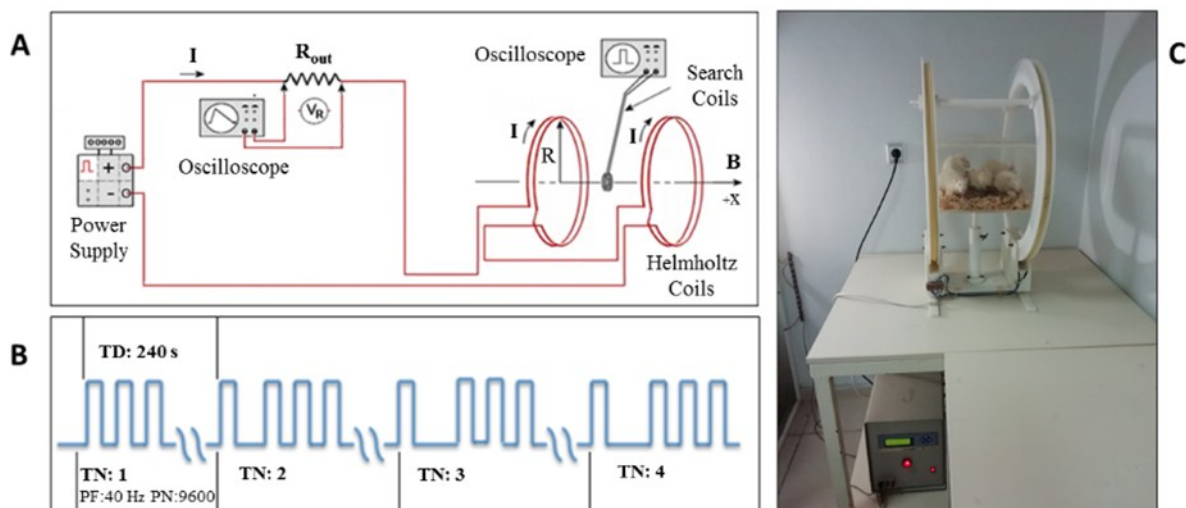
In the present study, 20 male Wistar albino rats (180–230 g) were obtained from Cukurova University Health Sciences Experimental Application and Research Center. Animals were kept in a controlled room (with stable climate and lighting) with free access to standard food pellets and water during the experimental procedures.

### Pulsed magnetic field treatment system

The properties of the PMF system and the application procedures were as described previously<sup>(15)</sup>. Briefly, the components of the PMF system (ILFA Electronics, Adana, Turkey) included a power source, and Helmholtz coils (HCs) (coils diameter:60 cm, distances between coils:30 cm, produced uniform magnetic field: 1.5 mT). The value of the produced magnetic field was measured using a gauss meter with a Hall Effect probe. Induced electric field was calculated (according to Faraday's Law) as between 0.59 – 0.61 V/m at the center of HCs. The inductive

current was calculated as 0.67 A. The induced electric field waveform was measured with a search coil probe (coil diameter was 50mm, and consisted of 50 turns copper wire) placed at the center of HCs. The induced voltage was monitored through an oscilloscope (Hitachi, Japan), connected to the probe leads. The induced electric field had unipolar rectangular waveform with a magnitude of 0.6 V/m between the coils. The current at the circuit (5.2 A) was also monitored through an oscilloscope from a serial 0.9 Ohm resistor, which connected the serial between power source and coil outputs (figure 1A and 1C).

The PMF system was placed in the Faraday cage. PMF was applied in three sequences and each sequence consisted of four consecutive pulse trains. The duration of each pulse train was 240 s and the interval between each pulse train was 60 s (figure1B). Animals in the PMF group were exposed to the magnetic field for 30 days, at the same time every day for 1 hour (1.5 mT and 40 Hz). This procedure was applied to rats by placing them in special cages made of 3 mm thick transparent plexiglass. Animals in the control group were also placed in the magnetic field system for 30 days but without any magnetic field exposure. During the process, the temperature (23-25° C) and humidity (40-60 %) were frequently monitored.



**Figure 1.** **A. Left,** A schematic demonstration of the PMF system. **Right,** Plexiglass cage placed at the center of the Helmholtz coils during the PMF application. **B.** A schematized PMF application procedure. PMF was applied in three sequences and each sequence consisted of four consecutive pulse trains (Train Number (TN): 1,2,3,4, Train duration (TD): 240 s, Pulse Frequency (PF):40 Hz, and Pulse Number (PN):9600). The duration of each pulse train is 240 s and the interval between each pulse train is 60 s. **C.** A diagram of the magnetic field procedure. Animals is placed at the center of Helmholtz coils and received 1.5 mT PMF.

### Smooth muscle cell isolation

Primary vascular smooth muscle cells (VSMC) were enzymatically isolated from the rat aorta with collagenase type 1 (1.00 mg/ml) and elastase pancreatic type 1 (0.25 mg/ml) as previously described<sup>(16)</sup>. Briefly, after the aorta was dissected, the endothelium was denuded, and the adventitia was removed. The aorta was incubated with the

collagenase type 1 (1.00 mg/ml) for 15 minutes at 37°C in a CO<sub>2</sub> incubator. Then aorta was minced with scissors, incubated with both enzymes for 1 hour at 37°C in a CO<sub>2</sub> incubator, and pipetted every 10 minutes during incubation. Cell suspensions were centrifuged at 3000 rpm for 10 minutes at 4°C. Then pellets were re-suspended in DMEM with 10% fetal calf serum and plated into 25 cm<sup>2</sup> tissue culture

flasks. Cells were kept in a 37 °C humidified incubator with 5% CO<sub>2</sub>. The media was changed on the 4<sup>th</sup> and 6<sup>th</sup> day after the enzymatic procedure and passaged on the 7<sup>th</sup> day with 80 – 90% confluence.

### **MTT assay**

VSMCs viability was measured by MTT assay. Cells from the second passage were seeded into 96 well culture plates at 2x10<sup>3</sup> density cells/well for the MTT assay until 70-80 % confluence (2-3 days). After reaching confluence, 0.1 mM, 0.5 mM, 1 mM, or 5 mM of H<sub>2</sub>O<sub>2</sub> was added to the wells, and cells were incubated for 1 h at 37 °C in a humidified incubator. Each treatment was replicated in 3 wells. After the incubation, the cell proliferation and cytotoxicity were determined via MTT assay kits (Boster Biological Technology, CA, USA). According to the manufacturer's instruction, 10 µL of the MTT labeling reagent was added to each well. After incubation of the cells at 37°C for 4 hours, 100 µL of Formazan solubilization solution was added to each well and mixed thoroughly. The absorbance was read at 600 nm with a microplate reader (Awareness Technology, USA) after overnight incubation of the cells. The values from each experimental group were divided by the control group mean value and presented as fold change compared to the control group (Con) in the bar graph.

### **Caspase 3 enzyme activity measurement**

Enzyme activity of the vascular cells was measured using the caspase-3 colorimetric assay. VSMCs from the second passages were used in the assay. To induce apoptosis, we incubated cells with 1 mM H<sub>2</sub>O<sub>2</sub> for an hour and kept the cells in a 37°C humidified incubator with 5% CO<sub>2</sub>. The cells were trypsinized and counted (2 x 10<sup>6</sup> cells) before adding the pre-cooled lysis buffer. Caspase-3 activity was measured using a commercial kit (R&D Systems, MN, USA). Briefly, cells were collected after centrifugation at 250 x g for 10 min. The cell pellets were suspended with the pre-cooled lysis buffer followed by incubated on ice for 10 min. The cell homogenates were centrifuged at 10,000 g for 1 min and the supernatants were then removed. The cell lysate (50 µl), reaction buffer with DTT (50 µl), and 5 µl caspase -3 colorimetric substrate (DEVD-pNA) were added to the 96-microplate. The plate was incubated at 37°C for 1 hour before reading. According to the assay, caspase-3 cleaves the amino acid motif DEVD conjugated p-nitroaniline to colored p-nitroaniline (pNA). The enzymatic activity was measured as the change in absorbance at 405 nm using a microplate reader (Awareness Technology, USA). The values from each group data were divided by the control group mean value and expressed as fold change compared to the control group (Con).

### **Western blotting**

Rat aortas were excised and randomly grouped as either H<sub>2</sub>O<sub>2</sub> treated or control. The H<sub>2</sub>O<sub>2</sub> treated group was incubated with H<sub>2</sub>O<sub>2</sub> for 1 h at 37°C oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) Krebs solution (mM): NaCl 115, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.17, CaCl<sub>2</sub> 2.5, NaHCO<sub>3</sub> 25, and glucose 5, pH: 7.4) and then stored at -80°C. Frozen tissues were pulverized in liquid nitrogen at -196°C and homogenized with a buffer solution containing 20 mM Tris-HCl, 150 mM NaCl, 2 mM KCl, 2 mM EDTA, 0.5 mM DTT, and 100 mM protease inhibitor cocktail, at pH 7.4. Homogenates were centrifuged at 1000 g for 10 min (4°C). The protein concentration of the supernatants was measured via the Bradford assay and caspase-3 expression was measured by Western blotting. Samples were run in SDS polyacrylamide gels (10%) under 20-40 mA constant current for 1.30 - 2 h. Then, protein bands were transferred to PVDF membranes and incubated overnight with caspase-3 antibodies (1/500 BosterBio, USA). B-actin antibodies (1/1000 BosterBio, USA) were used for the control of the internal loading of the proteins. The protein bands were revealed with ECL Western blotting substrate (Santa Cruz Biotechnology) and then visualized by autoradiography. The bands were quantified with ImageJ software (NIH, USA). The results were demonstrated as a fold change in the level of each protein normalized to B -actin from PMF and incubation groups compared to the control group.

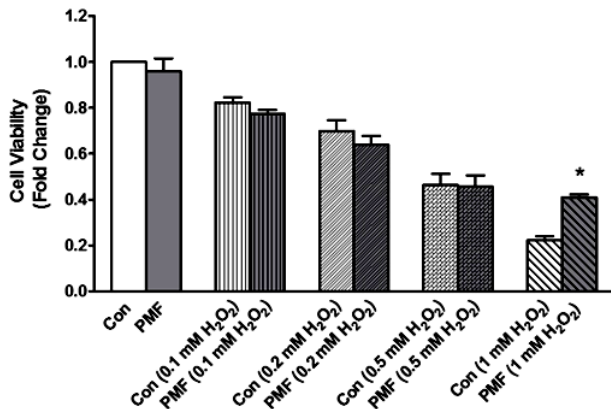
### **Statistical Analyses**

Appropriate statistical methods were performed using GraphPad Prism 5.0. (GraphPad Software, Inc, CA, USA).

## **RESULTS**

### **Effect of PMF-pre-application on VSMCs proliferation**

To investigate whether PMF pre-application has cytoprotective effects on H<sub>2</sub>O<sub>2</sub>-induced cell death, VSMCs were incubated with H<sub>2</sub>O<sub>2</sub> in different concentrations, and cell proliferation and viability were controlled by MTT assays. H<sub>2</sub>O<sub>2</sub> application decreased the viability in both control and PMF groups in a dose-dependent manner. However, cells isolated from PMF pre-applied rats (PMF groups) did not show any difference at low doses of H<sub>2</sub>O<sub>2</sub> (0.1 mM, 0.2 mM, and 0.5 mM) when compared to the control groups (such as control 0.1 mM and PMF 0.1 mM groups). However, the viability of the cells at 1 mM H<sub>2</sub>O<sub>2</sub> stayed higher in the PMF group when compared to its control counterpart (figure 2). Therefore, 1 mM H<sub>2</sub>O<sub>2</sub> was used in the subsequent experiments to reveal the effects of PMF pre-application at apoptosis,



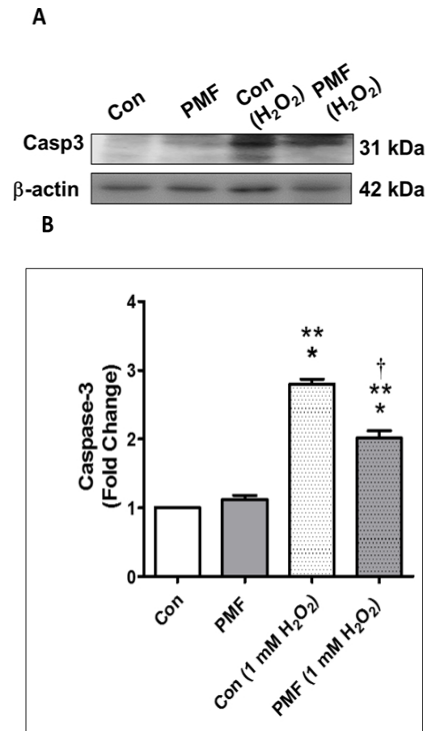
**Figure 2.** The effect of PMF on cell viability. VSMCs isolated from the PMF pre-applied (PMF groups) and control groups (Con groups) and incubated with or without H<sub>2</sub>O<sub>2</sub> (0.1, 0.2, 0.5, and 1 mM) for 1 h. The different concentration of hydrogen peroxide for each group is indicated in parentheses. Evaluation of PMF on cell viability using the MTT assay. Each group data is normalized to the control data. Bars represent mean  $\pm$  SEM, n = 8 animals/group (each triplicate). \* The difference between PMF (1mM) and Con (1mM) ( $p < 0.05$ ) as demonstrated using the student's t-test.

#### Effect of PMF on 1 mM H<sub>2</sub>O<sub>2</sub> induced apoptosis

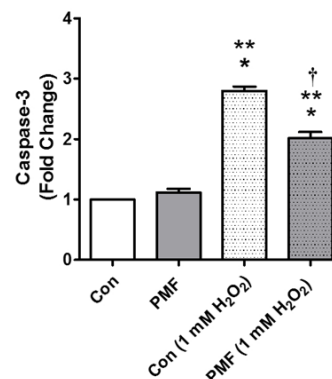
To examine if the increase in cellular viability in the 1 mM H<sub>2</sub>O<sub>2</sub> PMF group was due to a decrease in apoptosis, the caspase-3 protein level of the aortic tissues was investigated (figure 3A). Caspase-3 bands were normalized with  $\beta$ -actin bands to obtain bar graphic (figure 3B). As illustrated in figure. 3B, H<sub>2</sub>O<sub>2</sub> incubation for 1 h increased the caspase-3 levels when compared to the control group. However, pre-PMF application suppressed caspase-3 protein expression significantly in the tissues incubated with H<sub>2</sub>O<sub>2</sub> when compared to that of the control groups.

#### Caspase-3 activity

To identify whether the suppression of the protein levels in the PMF group is related to its caspase-3 activity, we performed the caspase-3 colorimetric assay. H<sub>2</sub>O<sub>2</sub> incubation for 1 h increased caspase-3 activity in the VSMCs. However, the PMF group had decreased caspase-3 activity when compared to its control group (figure 4).



**Figure 3.** The effect of PMF on caspase-3 expression in the rat aorta. **A.** Representative Western blots of caspase-3 and B-actin expressions from the PMF pre-applied (PMF) and control (Con) aorta homogenates, which are incubated with (Con (H<sub>2</sub>O<sub>2</sub>) and PMF (H<sub>2</sub>O<sub>2</sub>)) or without (Con and PMF) at 1 mM H<sub>2</sub>O<sub>2</sub>. **B.** Caspase-3 is normalized to B-actin. Comparison to the Con group. Bar graph shows mean  $\pm$  SEM, n = 5-6 homogenates/group, \* $p < 0.05$  versus Con, \*\* $p < 0.05$  versus PMF, † $p < 0.05$  versus Con (H<sub>2</sub>O<sub>2</sub>) as determined by one-way ANOVA.



**Figure 4.** The effect of PMF on caspase-3 activity. VSMCs were treated (Con (H<sub>2</sub>O<sub>2</sub>) and PMF (H<sub>2</sub>O<sub>2</sub>)) or without (Con and PMF) at 1 mM H<sub>2</sub>O<sub>2</sub> before assessing the caspase-3 activity. Each group data is normalized to the control. Bars represent mean  $\pm$  SEM, n = 8 animals/group (each performed triplicate), \* $p < 0.05$  versus Con, \*\* $p < 0.05$  vs PMF, † $p < 0.05$  vs Con (1mM) as determined by one-way ANOVA.

## DISCUSSION

PMF application is a non-invasive alternative treatment method for many diseases. However, the effect of PMF on vascular smooth muscle cells in oxidative stress conditions is unclear. Oxidative stress may lead to many cardiovascular diseases by impairing the apoptosis pathway<sup>(17,18)</sup>. Evidence from the literature suggests that PMF provides increased neovascularization and reendothelialization in the human vein (HUVEC) and bovine aortic endothelial cells<sup>(19)</sup>. Hence, the goal of the present study was to determine the protective effects of PMF pre-treatment on vascular smooth muscle cells during oxidative stress. According to the hypothesis of this study, PMF application has modulatory effects on cells, and these are revealed in regulated oxidative stress conditions. Our results provide the first evidence that *in vivo* PMF pre-treatment has protective effects on VSMCs at certain oxidative stress conditions. In this study, pre-exposure of 40 Hz PMF application for 30 days, increased VSMCs viability under 1 mM H<sub>2</sub>O<sub>2</sub> stimulation, while a similar effect was not observed in the presence of a lower H<sub>2</sub>O<sub>2</sub> dose.

In the present study, oxidative stress was induced by H<sub>2</sub>O<sub>2</sub> incubation. Previous studies have shown that H<sub>2</sub>O<sub>2</sub> is an endogenously released vasoactive agent and its effects on the vessel tonus are concentration-dependent<sup>(6,20)</sup>. Furthermore, past studies have shown that H<sub>2</sub>O<sub>2</sub> dose-dependently reduced cellular viability<sup>(21)</sup>, and 1 mM H<sub>2</sub>O<sub>2</sub> induced its maximum effect despite the presence of endogenous catalase activity<sup>(22)</sup>. In this study, isolated VSMCs were treated with four different H<sub>2</sub>O<sub>2</sub> concentrations for both control and pre-exposure PMF groups. According to MTT assays, H<sub>2</sub>O<sub>2</sub> decreased cell viability dose-dependently in VSMCs. However, in the PMF pre-exposure group, 1 mM H<sub>2</sub>O<sub>2</sub> had less effect on the viability of the cells when compared to the controls (1 mM H<sub>2</sub>O<sub>2</sub>). This is the first reported observation of the cellular protective effects of PMF pre-application in the vascular system at cytotoxic concentrations of H<sub>2</sub>O<sub>2</sub><sup>(23)</sup>.

Apoptosis plays a key role in the VSMCs cellular dysfunctions, leading to atherosclerotic plaque formation, thrombosis promotion, and plaque ruptures at the vessels<sup>(24,25,26,27,28)</sup>. Previous studies have demonstrated the effects of PMF on apoptosis and cell viability. Kumar *et al.* 2011 showed that PMF attenuated apoptosis parameters, such as caspase-3 induced with a 2.45-GHz microwave field.<sup>29</sup> In other studies, PMF increased apoptosis in only cancer cell lines. The application did not show apoptotic effects in non-cancer cells<sup>(10,30)</sup>. PMF treatment reduced the ER stress parameters (BiP, Grp94, and CHOP) of tunicamycin-treated HepG2 cells<sup>(31)</sup>. Moreover, PMF treatment had cytoprotective effects on H<sub>2</sub>O<sub>2</sub>-induced ROS responses of a neuroblastoma cell line<sup>(32)</sup>. In the

present study, H<sub>2</sub>O<sub>2</sub> stimulation increased the caspase-3 expression of VSMCs. However, pre-PMF treatment suppressed the caspase-3 protein level and decreased its enzymatic activities. The increased cell viability with decreased apoptotic activity in aortic vascular cells may signal a protective effect of PMF pre-application at the vasculature.

It was also shown that PMF application (15 and 72 Hz) for 1 or 6 h daily, for 25 days, affects vascular tissue growth and formation<sup>(33)</sup>. Bragin *et al.* (2015) observed that PMF application in healthy rats caused cerebral arteriolar dilation as demonstrated via *in-vivo* two-photon laser scanning microscopy. Moreover, intravenous L-NAME application reduced the vessel diameter that was directly related to the NO regulators during PMF treatment<sup>(34)</sup>. Changes in NO release may modulate apoptotic activities observed in various cell types<sup>(35,36)</sup>. The results of NO increase in the vascular components are complex. For example, it has been shown to inhibit VSMC proliferation, but induce endothelial cell promotion<sup>(37,38,39)</sup>. Therefore, in the present study, decreasing caspase-3 activity in PMF-treated VSMCs at H<sub>2</sub>O<sub>2</sub> incubation may be related to the PMF-NOS interplay. However, further studies are needed.

## CONCLUSION

In summary, our results showed for the first time that PMF pre-treatment has cytoprotective effects on VSMCs through reducing H<sub>2</sub>O<sub>2</sub>-induced apoptosis. Considering the protective effects of PMF, the application may be a potential alternative treatment method for preventing oxidative stress-related vascular system diseases.

## ACKNOWLEDGEMENTS

*none.*

**Conflict of interest:** none.

**Ethical approval:** Ethical approval: The study and all the animal procedures were approved and performed in accordance with the Cukurova University Local Ethics Committee on Animal Experiments standards (approval no: 31.01.2017/1)

**Funding:** none.

**Authors' contributions:** FC and IG conceived and designed the study. FC, IB and MT carried out the experiment. FC, CC and IB analyzed the data. FC, CC and IG wrote and revised the manuscript.

## REFERENCES

1. Kang DH and Kang SW (2013) Targeting cellular antioxidant enzymes for treating atherosclerotic vascular disease. *Biomol Ther (Seoul)*, **21**(2): 89-96.
2. Touyz RM, Schiffrin EL (2004) Reactive oxygen species in vascular biology: implications in hypertension. *Histochem Cell Biol*, **122**(4): 339-352.

3. Ardanaz N and Pagano PJ (2006) Hydrogen peroxide as a paracrine vascular mediator: regulation and signaling leading to dysfunction. *Exp Biol Med (Maywood)*, **231**(3): 237-251.
4. Touyz RM (2000) Oxidative stress and vascular damage in hypertension. *Curr Hypertens Rep*, **2**(1): 98-105.
5. Burtenshaw D, Kitching M, Redmond EM, Megson IL, Cahill PA (2019) Reactive Oxygen Species (ROS), Intimal Thickening, and Subclinical Atherosclerotic Disease. *Front Cardiovasc Med*, **6**: 89.
6. Thengchaisri N and Kuo L (2003) Hydrogen peroxide induces endothelium-dependent and -independent coronary arteriolar dilation: role of cyclooxygenase and potassium channels. *Am J Physiol Heart Circ Physiol*, **285**(6): H2255-2263.
7. Tatarov I, Panda A, Petkov D, Kolappaswamy K, Thompson K, Kavirayani A, Lipsky MM, Elson E, Davis CC, Martin SS, DeTolla LJ (2011) Effect of magnetic fields on tumor growth and viability. *Comp Med*, **61**(4): 339-345.
8. Kaszuba-Zwoinska J, Chorobik P, Juszcak K, Zaraska W, Thor PJ (2012) Pulsed electromagnetic field affects intrinsic and endoplasmic reticulum apoptosis induction pathways in MonoMac6 cell line culture. *J Physiol Pharmacol*, **63**(5): 537-545.
9. Kaszuba-Zwoinska J, Wojcik K, Bereta M, Ziomber A, Pierzchalski P, Rokita E, Marcinkiewicz J, Zaraska W, Thor P (2010) Pulsating electromagnetic field stimulation prevents cell death of puromycin treated U937 cell line. *J Physiol Pharmacol*, **61**(2): 201-205.
10. Crocetti S, Beyer C, Schade G, Egli M, Frohlich J, Franco-Obregon A (2013) Low intensity and frequency pulsed electromagnetic fields selectively impair breast cancer cell viability. *PLoS One*, **8**(9): e72944.
11. Zhang X, Zhang H, Zheng C, Li C, Zhang X, Xiong W (2002) Extremely low frequency (ELF) pulsed-gradient magnetic fields inhibit malignant tumour growth at different biological levels. *Cell Biol Int*, **26**(7): 599-603.
12. Wang H and Zhang X (2017) Magnetic Fields and Reactive Oxygen Species. *Int J Mol Sci*, **18**(10).
13. Wang C, Liu Y, Wang Y, Wei Z, Suo D, Ning G, Wu Q, Feng S, Wan C (2019) Low frequency pulsed electromagnetic field promotes functional recovery, reduces inflammation and oxidative stress, and enhances HSP70 expression following spinal cord injury. *Mol Med Rep*, **19**(3): 1687-1693.
14. Falone S, Marchesi N, Osera C, Fassina L, Comincini S, Amadio M, Pascale A (2016) Pulsed electromagnetic field (PEMF) prevents pro-oxidant effects of H<sub>2</sub>O<sub>2</sub> in SK-N-BE(2) human neuroblastoma cells. *Int J Radiat Biol*, **92**(5): 281-286.
15. Gunay I and Mert T (2011) Pulsed magnetic fields enhance the rate of recovery of damaged nerve excitability. *Bioelectromagnetics*, **32**(3): 200-208.
16. Lo Russo A, Passaquain AC, Andre P, Skutella M, Rugg UT (1996) Effect of cyclosporin A and analogues on cytosolic calcium and vasoconstriction: possible lack of relationship to immunosuppressive activity. *Br J Pharmacol*, **118**(4): 885-892.
17. Mallat Z and Tedgui A (2000) Apoptosis in the vasculature: mechanisms and functional importance. *Br J Pharmacol*, **130**(5): 947-962.
18. Stoneman VE and Bennett MR (2004) Role of apoptosis in atherosclerosis and its therapeutic implications. *Clin Sci (Lond)*, **107**(4): 343-354.
19. Yen-Patton GP, Patton WF, Beer DM, Jacobson BS (1998) Endothelial cell response to pulsed electromagnetic fields: stimulation of growth rate and angiogenesis in vitro. *J Cell Physiol*, **134**(1): 37-46.
20. Fujimoto S, Mori M, Tsushima H (2003) Mechanisms underlying the hydrogen peroxide-induced, endothelium-independent relaxation of the norepinephrine-contraction in guinea-pig aorta. *Eur J Pharmacol*, **459**(1): 65-73.
21. Xu Y, Kang J, Yuan Z, Li H, Su J, Li Y, Kong X, Zhang H, Wang W, Sun L (2013) Suppression of CLIC4/mtCLIC enhances hydrogen peroxide-induced apoptosis in C6 glioma cells. *Oncol Rep*, **29**(4): 1483-1491.
22. Pelaez NJ, Braun TR, Paul RJ, Meiss RA, Packer CS (2000) H<sub>2</sub>O<sub>2</sub> mediates Ca<sup>2+</sup>- and MLC(20) phosphorylation-independent contraction in intact and permeabilized vascular muscle. *Am J Physiol Heart Circ Physiol*, **279**(3): H1185-1193.
23. Gulden M, Jess A, Kammann J, Maser E, Seibert H (2010) Cytotoxic potency of H<sub>2</sub>O<sub>2</sub> in cell cultures: impact of cell concentration and exposure time. *Free Radic Biol Med*, **49**(8): 1298-1305.
24. Bauriedel G, Hutter R, Welsch U, Bach R, Sievert H, Luderitz B (1999) Role of smooth muscle cell death in advanced coronary primary lesions: implications for plaque instability. *Cardiovasc Res*, **41**(2): 480-488.
25. Clarke M and Bennett M (2006) Defining the role of vascular smooth muscle cell apoptosis in atherosclerosis. *Cell Cycle*, **5**(20): 2329-2331.
26. Clarke M.C, Figg N, Maguire JJ, Davenport AP, Goddard M, Littlewood TD, Bennett MR (2006) Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nat Med*, **12**(9): 1075-1080.
27. Flynn P D, Byrne CD, Baglin TP, Weissberg PL, Bennett MR (1997) Thrombin generation by apoptotic vascular smooth muscle cells. *Blood*, **89**(12): 4378-4384.
28. Wang P, Xu TY, Guan YF, Zhao Y, Li ZY, Lan XH, Wang X, Yang PY, Kang ZM, Vanhoutte PM, Miao CY (2014) Vascular smooth muscle cell apoptosis is an early trigger for hypothyroid atherosclerosis. *Cardiovasc Res*, **102**(3): 448-459.
29. Kumar S, Kesari KK, Behari J (2011) The therapeutic effect of a pulsed electromagnetic field on the reproductive patterns of male Wistar rats exposed to a 2.45-GHz microwave field. *Clinics (Sao Paulo)*, **66**(7): 1237-1245.
30. Filipovic N, Djukic T, Radovic M, Cvetkovic D, Curcic M, Markovic S, Peulic A, Jeremic B (2014) Electromagnetic field investigation on different cancer cell lines. *Cancer Cell International*, **14**.
31. Keczan E, Keri G, Banhegyi G, Stiller I (2016) Effect of pulsed electromagnetic fields on endoplasmic reticulum stress. *J Physiol Pharmacol*, **67**(5): 769-775.
32. Osera C, Amadio M, Falone S, Fassina L, Magenes G, Amicarelli F, Ricevuti G, Govoni S, Pascale A (2015) Pre-exposure of neuroblastoma cell line to pulsed electromagnetic field prevents H<sub>2</sub>O<sub>2</sub>-induced ROS production by increasing MnSOD activity. *Bioelectromagnetics*, **36**(3): 219-232.
33. Greenough CG (1992) The effects of pulsed electromagnetic fields on blood vessel growth in the rabbit ear chamber. *J Orthop Res*, **10**(2): 256-262.
34. Bragin DE, Statom GL, Hagberg S, Nemoto EM (2015) Increases in microvascular perfusion and tissue oxygenation via pulsed electromagnetic fields in the healthy rat brain. *J Neurosurg*, **122**(5): 1239-1247.
35. Dimmeler S, Haendeler J, Nehls M, Zeiher AM (1997) Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J Exp Med*, **185**(4): 601-607.
36. Mannick JB, Miao XQ, Stamler JS (1997) Nitric oxide inhibits Fas-induced apoptosis. *J Biol Chem*, **272**(39): 24125-24128.
37. Brune B (2003) Nitric oxide: NO apoptosis or turning it ON? *Cell Death Differ*, **10**(8): 864-869.
38. Jeremy JY, Rowe D, Emsley AM, Newby AC (1999) Nitric oxide and the proliferation of vascular smooth muscle cells. *Cardiovasc Res*, **43**(3): 580-594.
39. Levine AB, Punihaole D, Levine TB (2012) Characterization of the role of nitric oxide and its clinical applications. *Cardiology*, **122**(1): 55-68.