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

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► ***Original article***

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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 [1,2]. 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 [3,4]. 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 [2,5,6]. 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 [7]. In monocytic cell lines, a pulsed magnetic field (PMF) increased the Bcl-2 anti-apoptotic protein expression via the caspase-independent pathway [8]. In another study, the application decreased apoptosis in the U397 human lymphoid cell line treated with puromycin, a telomerase and apoptosis inhibitor [9]. 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 [10]. Furthermore, previous data have demonstrated that in vivo PMF application inhibits tumor cell growth via inducing apoptosis and blocking neovascularisation formation in tumor development [11].

The protective effect of PMF on oxidative stress was also shown in previous studies [12]. PMF application at 2.5 mT and 50 Hz reduced oxidative stress following spinal cord injury [13]. 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 [14]. 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.

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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 [15]. 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 vith 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.

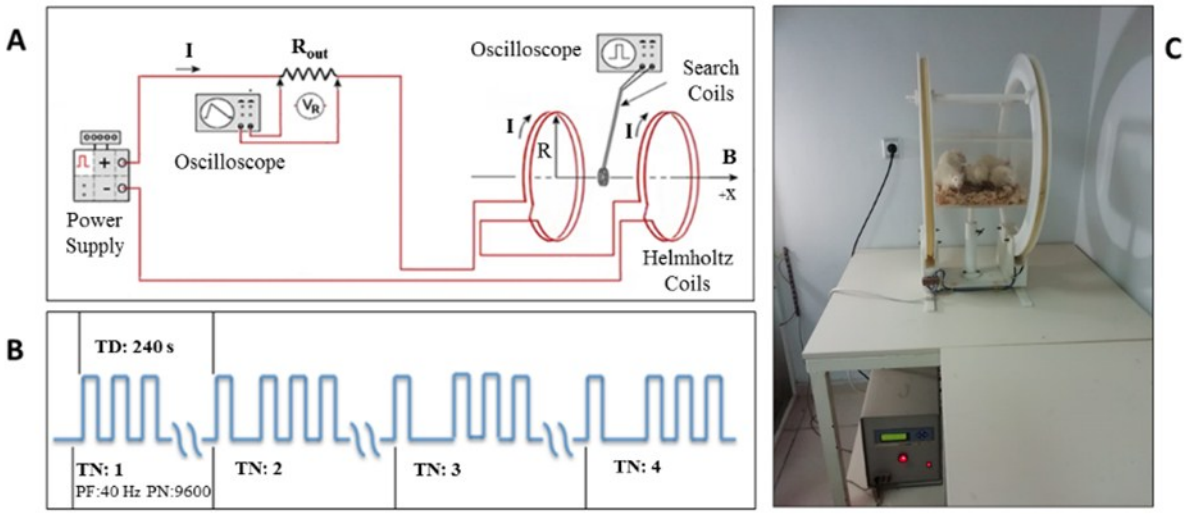


Figure 1. A. Left, A schematic demonstration of the PMF system. **Right**, Plexiglass cage placed at the center of the Helmholtz coils during the PMF application. **B.** A schematized PMF application procedure. PMF was applied in three sequences and each sequence consisted of four consecutive pulse trains (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 [16]. 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_2 incubator. Then aorta was minced with scissors, incubated with both enzymes for 1 hour at 37ºC in a CO_2 incubator, and pipetted every 10 minutes during incubation. Cell suspensions were centrifuged at 3000 rpm for 10 minutes at 4ºC. Then pellets were re-suspended in DMEM with 10% fetal calf serum and plated into 25 cm^2 tissue culture

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flasks. Cells were kept in a 37 °C humidified incubator with 5% CO_2. The media was changed on the 4th and 6th day after the enzymatic procedure and passaged on the 7th 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 $2x10^3$ 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_2O_2 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_2O_2 for an hour and kept the cells in a 37°C humidified incubator with 5% CO_2. The cells were trypsinized and counted (2 x 10^6 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_2O_2 treated or control. The H_2O_2 treated group was incubated with H_2O_2 for 1 h at 37°C oxygenated (95% O_2 and 5% CO_2) Krebs solution (mM): NaCl 115, KCI 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaC1_2$ 2.5, $NaHCO_3$ 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_2O_2-induced cell death, VSMCs were incubated with H_2O_2 in different concentrations, and cell proliferation and viability were controlled by MTT assays. H_2O_2 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_2O_2 (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_2O_2 stayed higher in the PMF group when compared to its control counterpart (figure 2). Therefore, 1 mM H_2O_2 was used in the subsequent experiments to reveal the effects of PMF pre-application at apoptosis,

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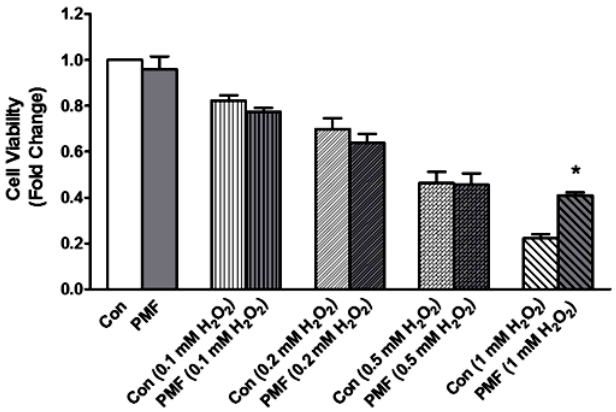


Figure 2. The effect of PMF on cell viability. VSMCs isolated from the PMF pre-applied (PMF groups) and control groups (Con groups) and incubated with or without H2O2 (0.1, 0.2, 0.5, and 1 mM) for 1 h. The different concentration of hydrogen peroxide for each group is indicated in parentheses. Evaluation of PMF on cell viability using the MTT assay. Each group data is normalized to the control data. Bars represent mean ± 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_2O_2 induced apoptosis

To examine if the increase in cellular viability in the 1 mM H_2O_2 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 β-actin bands to obtain bar graphic (figure 3B). As illustrated in figure. 3B, H_2O_2 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_2O_2 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_2O_2 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).

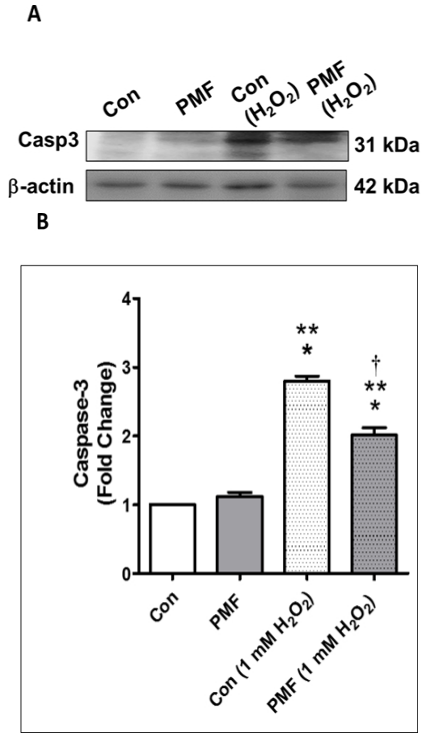


Figure 3. The effect of PMF on caspase-3 expression in the rat aorta. **A.** Representative Western blots of caspase-3 and B-actin expressions from the PMF pre-applied (PMF) and control (Con) aorta homogenates, which are incubated with (Con (H2O2) and PMF (H2O2)) or without (Con and PMF) at 1 mM H2O2. **B.** Caspase-3 is normalized to B-actin. Comparison to the Con group. Bar graph shows mean ± SEM, n = 5-6 homogenates/group, *$p < 0.05$ versus Con, **$p < 0.05$ versus PMF, †$p < 0.05$ versus Con (H2O2) as determined by one-way ANOVA.

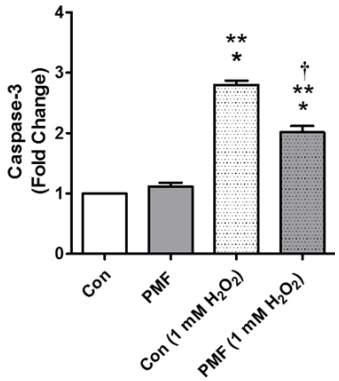


Figure 4. The effect of PMF on caspase-3 activity. VSMCs were treated (Con (H2O2) and PMF (H2O2)) or without (Con and PMF) at 1 mM H2O2 before assessing the caspase-3 activity. Each group data is normalized to the control. Bars represent mean ± SEM, n = 8 animals/group (each performed triplicate), *$p< 0.05$ versus Con, **$p < 0.05$ vs PMF, †$p < 0.05$ vs Con (1mM) as determined by one-way ANOVA.

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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 [17,18]. Evidence from the literature suggests that PMF provides increased neovascularization and reendothelialization in the human vein (HUVEC) and bovine aortic endothelial cells [19]. 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_2O_2 stimulation, while a similar effect was not observed in the presence of a lower H_2O_2 dose.

In the present study, oxidative stress was induced by H_2O_2 incubation. Previous studies have shown that H_2O_2 is an endogenously released vasoactive agent and its effects on the vessel tonus are concentration-dependent [6,20]. Furthermore, past studies have shown that H_2O_2 dose-dependently reduced cellular viability [21], and 1 mM H_2O_2 induced its maximum effect despite the presence of endogenous catalase activity [22]. In this study, isolated VSMCs were treated with four different H_2O_2 concentrations for both control and pre-exposure PMF groups. According to MTT assays, H_2O_2 decreased cell viability dose-dependently in VSMCs. However, in the PMF pre-exposure group, 1 mM H_2O_2 had less effect on the viability of the cells when compared to the controls (1 mM H_2O_2). This is the first reported observation of the cellular protective effects of PMF pre-application in the vascular system at cytotoxic concentrations of H_2O_2 [23].

Apoptosis plays a key role in the VSMCs cellular dysfunctions, leading to atherosclerotic plaque formation, thrombosis promotion, and plaque ruptures at the vessels [24,25,26,27,28]. 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.[29] In other studies, PMF increased apoptosis in only cancer cell lines. The application did not show apoptotic effects in non-cancer cells [10,30]. PMF treatment reduced the ER stress parameters (BiP, Grp94, and CHOP) of tunicamycin-treated HepG2 cells [31]. Moreover, PMF treatment had cytoprotective effects on H_2O_2-induced ROS responses of a neuroblastoma cell line [32]. In the present study, H_2O_2 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 [33]. 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 [34]. Changes in NO release may modulate apoptotic activities observed in various cell types [35,36]. 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 [37,38,39]. Therefore, in the present study, decreasing caspase-3 activity in PMF-treated VSMCs at H_2O_2 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_2O_2-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.

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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)

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Authors' contributions: FC and IG conveinced 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.

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