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Pulsed magnetic field maintains vascular homeostasis against H_2O_2-induced oxidative stress

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Abstract. Pulsed magnetic fields (PMFs) have significant therapeutic effects on many disorders. However, the effects of PMF on vascular homeostasis remain unclear. Therefore, in the present study, we investigated the role of *in vivo* PMF in maintaining vascular homeostasis during H_2O_2-induced oxidative stress. For this purpose, rats were exposed to PMF (40 Hz, 1.5 mT) for 1 h for a period of 30 days, following which their thoracic aortas were excised. H_2O_2 was exogenously applied to the aortic rings. Constrictions were measured in a tissue bath using an electrophysiological technique. Bcl-2 and endothelial nitric oxide synthase (eNOS) protein levels were determined by Western blotting. We found lesser H_2O_2-induced vasoconstriction in the PMF group than in the control group in endothelium-intact (E+) rings. As H_2O_2 also induces apoptosis, after incubation with H_2O_2 (40 min) to induce early apoptosis, we added KCl and measured KCl-induced contractions. All the groups, endothelium intact or denuded (E-) showed decreased responses; however, we still observed the effect of PMF in the E+ group due to increased endothelial activity. In addition, PMF increased the expression of the eNOS protein, which might be a key target of PMF. Our results suggest that *in vivo* application of PMF protects vascular responses through endothelium-mediated mechanisms during oxidative stress. Therefore, PMF might play a protective role against vascular diseases.

Key words: Pulsed magnetic fields — H_2O_2 — Thoracic aorta — Endothelium — Oxidative stress

Introduction

The therapeutic effects of pulsed magnetic fields (PMFs) have been reported in many experimental and clinical studies (Trock et al. 1993, 1994; Scardino et al. 1998; Pipitone and Scott 2001; Callaghan et al. 2008; Assiotis et al. 2012). In particular, PMF exposure has been found to increase the healing rates of bone fractures and wounds in both humans and animals (Chalidis et al. 2011; Pesce et al. 2013), improve wound epithelialization in dogs (Scardino et al. 1998), and prevent tissue necrosis in diabetic mice (Callaghan et al. 2008; Morabito et al. 2010; Crocetti et al. 2013; Filipovic et al. 2014). Moreover, the ameliorating effects of PMF have been observed in the aorta of diabetic rats (Ocal et al. 2018). The effects of PMF on apoptosis have been examined in cancer studies. Crocetti et al. (2013) showed that PMF inhibits the proliferation of MCF7 (a human breast adenocarcinoma cell line) but not that of MCF10 (a breast epithelial cell line). PMF exposure has been found to induce apoptosis and decrease proliferation and neuritogenesis in various cancer cell lines, such as SW-480 (colon cancer) and PC12 (pheochromocytoma) (Morabito et al. 2010; Filipovic et al. 2014). Cancer studies performed by injecting cancer cells, such as the mouse breast tumor cell line and B16 murine melanoma cells, in mice *in vivo* have shown that PMF inhibits tumor expansion (Yen-Patton et al. 1988; Nuccitelli et al. 2006; Tatarov et al. 2011; Vadala et al. 2016). The effects of PMF application have also been investigated using different cell types. PMF has been found to enhance human umbilical vein endothelial cell (HUVEC) proliferation, without affecting osteoblasts and fibroblasts (Tepper et al. 2004). Another study involving HUVECs revealed that PMF increases cell vascularization (Yen-Patton et al. 1988).

Although the effects of PMF have been identified using different cell types and disease models, its contribution to the vascular system remains unclear. Therefore, in the present study, we examined the effect of *in vivo* PMF treatment on the aorta *via* electrophysiological and molecular analyses. We measured vasoconstrictions in the excised aortas using

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high-dose (1 mM) H_2O_2, a reactive oxygen species (ROS) that is released from the endothelium at almost mM levels in some inflammatory conditions (Suematsu et al. 1993). H_2O_2 modulates constrictions by impairing endothelium-released relaxation factors, such as nitric oxide (NO), or *via* smooth muscle constriction mechanisms, depending on its concentration (Ardanaz and Pagano 2006; Santiago et al. 2013; Silva et al. 2017). To identify the effect of PMF, we used endothelium-intact (E+) and endothelium-denuded (E−) aortas in the present study. Moreover, we examined the effect of PMF application on endothelial nitric oxide synthase (eNOS) protein levels in order to identify its efficacy in regulating NO and thereby vascular smooth muscle constrictions. In addition, as H_2O_2 is an apoptotic agent, we investigated the constrictions at apoptosis on incubation with H_2O_2. We controlled apoptosis by examining Bcl-2 protein levels. Furthermore, we assessed whether the effect of PMF is associated with a different constriction mechanism, such as G protein-coupled receptor activation or membrane depolarization; we used Phe (phenylephrine) and KCl, respectively, to induce these pathways. Overall, our findings sign the role of PMF in maintaining vascular homeostasis which reveals with oxidative damage in the rat aorta.

Materials and Methods

The present study was approved by the Cukurova University Local Ethics Committee on Animal Experiments (No: 9/27.10.2016). Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

PMF system

The PMF system and exposure protocol used in the present study have been described previously (Mert et al. 2006; Gunay and Mert 2011). In brief, the PMF system (ILFA Electronics, Adana, Turkey) consists of a power source and a pair of Helmholtz coils (HCs) (60 cm diameter, 18-gauge copper wire with 100 turns) 30 cm apart from each other that produce a uniform magnetic field in a Faraday cage and a 1.5 mT magnetic field through a signal generator (Fig. 1A). The peak value of the generated magnetic field was measured using a Gauss meter with a Hall Effect probe (F.W. Bell Model 6010, Sypris, Orlando, FL). The magnetic field had a quasi-triangular waveform (rise time: 0.5 ms, fall time: 9.5 ms). The induced electric field and the waveform were measured using

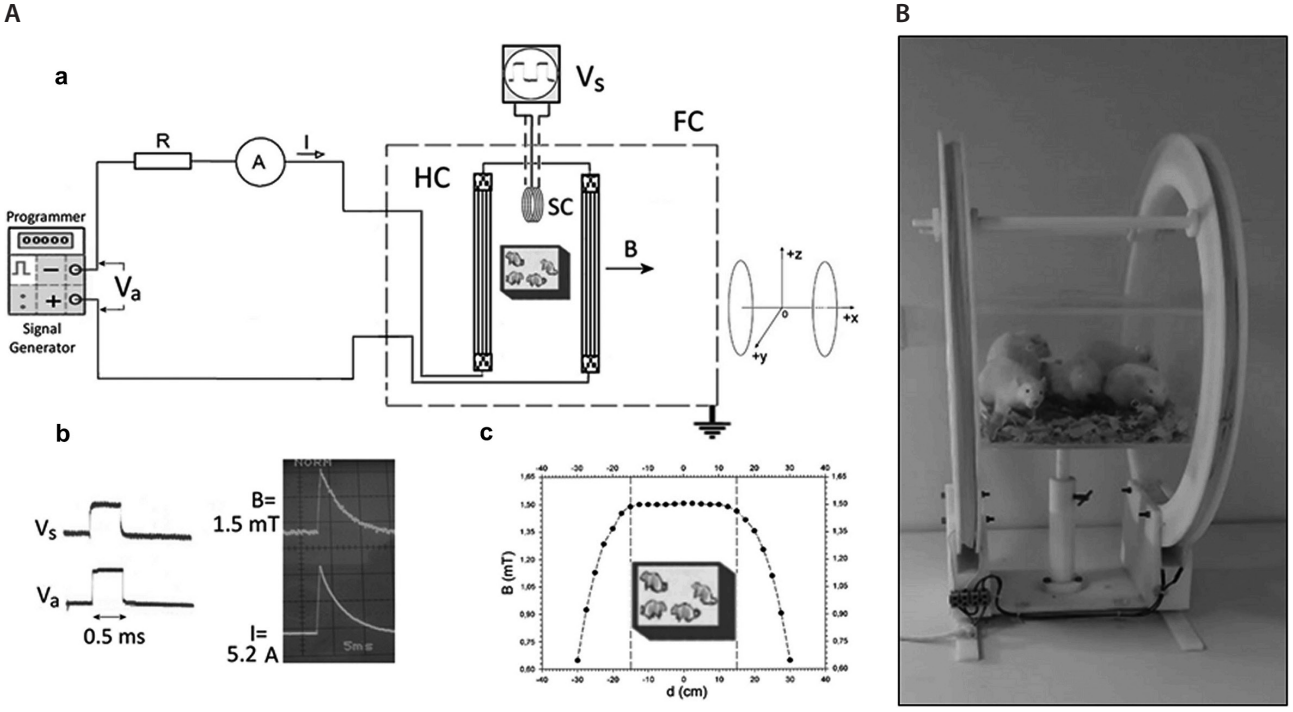

Figure 1. Pulsed magnetic field (PMF) application system. **A.a.** The magnetic field was set *via* a programmer. Rectangular voltage pulses (V_a) were applied through a signal generator. A triangular current (I = 5.2 A) was passed through the Helmholtz coils (HC), and a quasi-triangular 1.5 mT magnetic field (B) was formed between the HC. In the HC, the magnetic field was measured using a teslameter (**b**). Moreover, the induced voltage was observed using a search coil (SC) as V_s. **c.** Spatial variation of PMF in the y-z plane between the HCs. Rats were placed in a plexiglas cage where the magnetic field was constant. The current (I) was measured on the resistor R. FC, Faraday cage (earthed). **B.** During PMF application, 4–5 rats were placed in a plexiglas cage inside the HCs.

a search coil (0.5 cm diameter, 30-gauge copper wire with 50 turns) placed at the center of the HCs. The search coil was connected to an oscilloscope, and the corresponding peak electric field was set at 0.6 V/m with a rectangular waveform. The maximum value of the induced electric field was calculated on the basis of Faraday's law. The electric field varied between 0.59 and 0.61 V/m in the center of HCs, where the experimental plexiglas cage was located. The inductive current in the coil was calculated as 5.2 A from the voltage drop through a serially connected resistor (0.9 Ω) in the system.

Animal groups and PMF exposure protocol

Male Wistar albino rats were used in the experiments. The animals were randomly divided into 2 groups, each containing 9–10 rats. In the middle center of the HCs 4–5 rats were placed in a 30×30×20 cm plexiglas cage with 3 mm thickness without anesthesia procedure (Fig. 1B). The PMF-applied group (PMF group) was exposed to PMF for 1 h for a period of 30 days. The control group was placed in the magnetic field system for 30 days without being exposed to any magnetic field, as sham. Application was performed at the same time during the experiments. The temperature (23–25°C) and humidity (40–60%) of the Faraday cage were kept stable for both the groups.

PMF was applied as 4 consecutive pulse trains at 40 Hz. The duration of each pulse train was 4 min, with a 1-min interval between the pulse trains. We defined the PMF parameters according to those used in previous studies conducted at our laboratory. In those studies, the frequency ranged between 1 Hz and 40 Hz and the intensity was 1.5 mT; their effects were observed on diseases, such as neuropathy and diabetes mellitus (Mert et al. 2010; Gunay and Mert 2011).

Vascular activity measurements

The animals were anesthetized using pentobarbital sodium (30 mg/kg), following which their thoracic aorta were dissected, cut into 2–3 mm rings, and prepared as described previously (Zeydanli and Turan 2009). Two groups were further formed on the basis of the aortic rings: endothelium-intact (E+) and endothelium-denuded (E–) groups. In the E– group, the aortic ring lumens were rubbed gently with the tip of forceps to remove the endothelium. The rings were then hung on to isometric force transducers in a tissue bath containing Krebs solution (112 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11.5 mM glucose; pH 7.4), which was aerated with 95% O_2 and 5% CO_2 at 37°C**.** In the E+ group, the aortic rings were tested for a functional endothelium using acetylcholine (10^{-6} M) for relaxation after Phe contraction (10^{-5} M) (Zeydanli et al. 2010). The rings were stabilized for 60 min with 1 g of passive tension, and their contractility was examined using 60 mM KCl**.** The responses were recorded using the MP35 Data Acquisition System (Biopac System, USA).

Western blotting

The excised aortas were 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. The samples were centrifuged at 1000 × g (10 min, −4°C), and their supernatants were obtained. The protein concentration of the samples was measured by the Bradford assay (Pierce Biotechnology, USA). The expression levels of Bcl-2 and eNOS proteins were determined by Western blotting. The samples were loaded on SDS polyacrylamide gels (10%) under 20–40 mA constant current for 2 h. Protein bands were transferred to PVDF membranes and incubated overnight at 4°C with eNOS antibody (1:500, Bosterbio, USA) or Bcl-2 antibody (1:500, Cell Signaling, USA). β-actin (1/1000, Bosterbio, USA) was used as the internal loading control for the proteins.

Chemicals

All the chemicals used in the experiments were obtained from Sigma Aldrich, except where stated otherwise.

Statistics

The analyzed data are presented as the mean ± SD. Concentration-response curves and the half-maximal effective concentration (log EC_{50}) were obtained by curve-fitting. One-way ANOVA was used to identify statistical significance (at $p < 0.05$). Statistical analysis was performed using GraphPad Prism 5.

Results

Monitoring the effects of in vivo PMF on vasoconstrictions H_2O_2-induced contractions

H_2O_2 is an endogenously released vasoconstrictor in the vascular system. In the present study, exogenously applied H_2O_2 (1 mM) transiently constricted the aortic tissues. H_2O_2-induced contractions were followed by rapid relaxation (Fig. 2A). The difference in the H_2O_2-induced contractions was not statistically significant between the E+ and E– control groups. The lack of difference in constriction between the E+ and E– control groups on H_2O_2 stimulation indicates the absence of endothelium-mediated relaxation, resulting in functional impairment known as endothelial dysfunction. Impaired endothelial functioning is a result of reduced efficacy of endothelium-released relaxation factors, causing impaired functioning of blood vessels (Bonetti et al. 2003).

However, we observed lesser constrictions in the PMF E+ group than in the Control E+ group. Furthermore, we

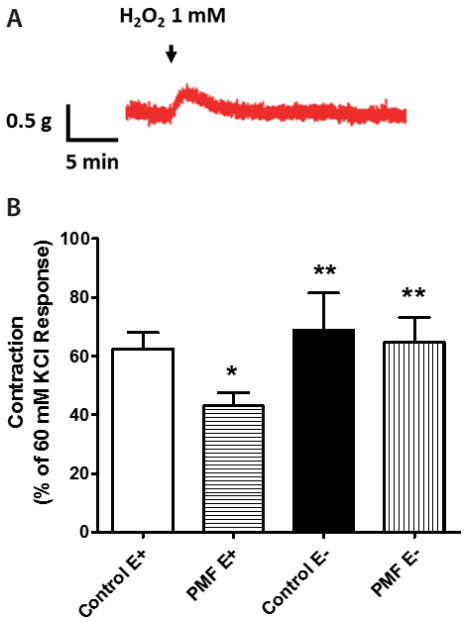

Figure 2. Contractions of aortic rings induced by 1 mM H_2O_2. **A.** A representative trace shows the typical aortic contraction induced by exogenously applied H_2O_2 (expressed in gram-force units per minute). **B.** Data show the differences in H_2O_2-induced contractions of E+ and E– aortic rings in the control and PMF groups. Results are expressed as the percentage of maximal contraction induced by 60 mM KCl. Data represented as the mean ± SD, $n = 9–10$ rats/group. * $p < 0.05$ *vs.* Control E+ group, ** $p < 0.05$ *vs.* PMF E+ group (by one-way ANOVA).

observed that the effect of the endothelium on contractions was preserved in the E+ PMF group, with a statistically significant difference being observed between the Control E– and PMF E– groups. The decreased response in the PMF E+ group indicates a more active endothelium-mediated response mechanism due to PMF application.

No difference was noted between the PMF E– and Control E– groups (Fig. 2B).

Effect of PMF application on H_2O_2-induced apoptosis

To observe the effect of PMF application on apoptosis, we incubated the aortic rings with 1 mM H_2O_2 for 40 min in a tissue bath and froze them until use. After thawing and homogenizing the samples, we assessed Bcl-2 protein expression by Western blotting to control the apoptosis. As shown in Figure 3A and B, the anti-apoptotic Bcl-2 protein bands completely disappeared on incubation with 1 mM H_2O_2 in the PMF and control groups, indicating the initiation of apoptosis in the tissues. Incubation with 1 mM H_2O_2 (30–60 min) has been found to induce apoptosis and decrease vasoconstriction induced by the subsequently applied agonist in the aorta (Mian and Martin 1997). In our experiments, we also impaired the vascular tissue metabolism *via* H_2O_2-induced apoptosis. According to our hypothesis, the responses of the PMF group to impaired vascular functions could vary from those of the control group due to modulations occurring during PMF application. Therefore, during the H_2O_2-induced apoptosis, we investigated the responses of the PMF group to constrictions induced by the subsequently applied agonist, KCl. As shown in Figure 3C, during incubation with H_2O_2, we added KCl and examined the resulting contractions. In brief, after adding 1 mM H_2O_2 into the chamber and incubating the rings for 40 min with H_2O_2, we added 60 mM KCl into the incubation solution and com-

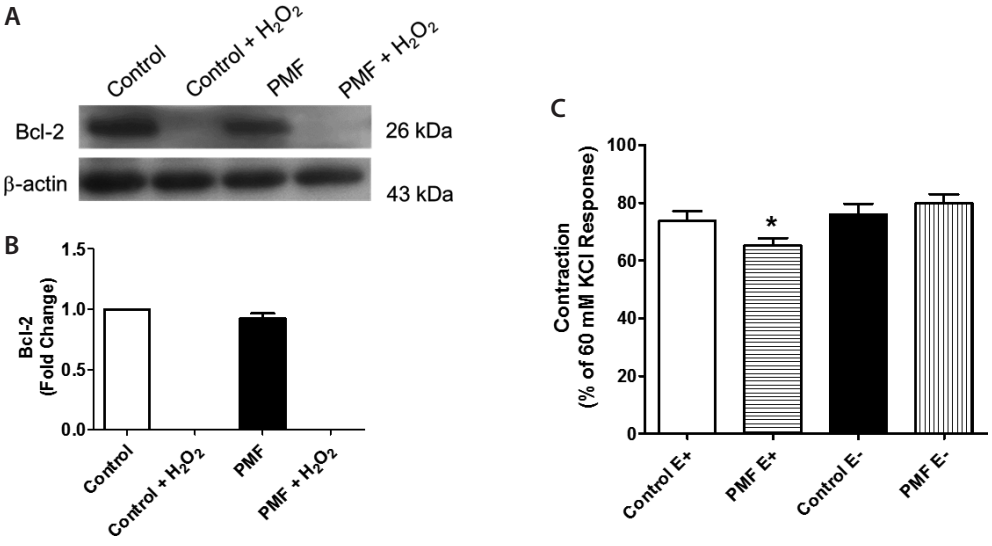

Figure 3. Effect of PMF on regulation of apoptosis in the rat aorta. Apoptosis was induced in the aortic rings by incubation with 1 mM H_2O_2 for 40 min. **A.** Western blot data representing Bcl-2 and β-actin expression levels in the control group, control group incubated with H_2O_2, PMF group, and PMF group incubated with H_2O_2. **B.** The graph indicates Bcl-2 expression levels between the groups. The band values were normalized to corresponding β-actin band values for each group. **C.** After H_2O_2 incubation, the rings were contracted with KCl in the E+ and E– control groups along with their corresponding PMF groups. Data are expressed as the percentage of maximal contraction induced by 60 mM KCl. Bar graph represents the mean ± SD, $n = 9–10$ rats/group. * $p < 0.05$ *vs.* Control E+ group (by one-way ANOVA).

pared KCl-induced contractions between the groups. After incubation with H_2O_2, we observed decreased KCl-induced constrictions in all the groups. The response to KCl did not differ between the PMF E– and Control E– groups. However, significantly lesser constriction was observed in the PMF E+ group. This indicates that PMF application has a modulating action in endothelial functioning in the thoracic aorta.

Effect of PMF application on eNOS expression

To demonstrate the role of PMF application on the endothelium at the molecular level, we assessed eNOS protein expression in E+ tissues (Fig. 4A). As shown in Figure 4B, we normalized eNOS bands to β-actin, quantified the bands with image analysis software (ImageJ, USA), and compared the results in the PMF group with those in the control group (shown in the bar graph). Significantly higher eNOS expression was observed in the E+ PMF group. Thus, eNOS plays a prominent role in NO regulation; the increased eNOS protein expression in the aorta indicates a dynamic interference of PMF in the blood vessels.

Phe- and KCl-induced contractions

To assess whether the observed effect of PMF application is specific to H_2O_2-induced oxidative stress, we constricted the aortic rings through other physiological stimulations, such as receptor stimulation or membrane depolarization. Phe, an α1 adrenergic receptor agonist, was cumulatively added (10^{-9}–10^{-5} M) to the bath solution; however, no difference was observed in both E+ and E– tissues between the control and PMF groups (Fig. 5A and B). The log EC_{50} value of the E+ control and PMF groups was −7.14 ± 0.2 and −7.33 ± 0.3, respectively, while that of the E– control and PMF groups was −7.58 ± 0.5 and −7.8 ± 0.5, respectively. We also studied the effect of PMF on KCl-induced contractions. No difference was observed on KCl stimulation in both E+ (Fig. 5C) and E– (Fig. 5D) PMF groups; similar results were observed on Phe stimulation. Both Phe and KCl resulted in higher contractions in the E– group than in the E+ group because of the lack of eNOS activation (Feletou et al. 2012).

Discussion

The present study aimed to investigate the effects of *in vivo* PMF application on vascular functions by measuring aortic vasoactivity. PMF plays a role in modulating the vascular system homeostasis, as exhibited through endothelial mediated vasoconstrictions stimulated with H_2O_2, an oxidative stress inducer and vasoconstrictor (Thengchaisri and Kuo 2003; Arora et al. 2010; Hopkins 2013). Therefore, we focused on stimulations using H_2O_2, an important endogenously released ROS in the aorta. When oxygen is metabolized in the cell, ROS are released as superoxide anion (O_2^-) and H_2O_2 (Arora et al. 2010). In physiological homeostasis, the formation and elimination of oxidants are balanced. However, an imbalance between prooxidants and antioxidants occurs during oxidative stress, which can lead to cardiovascular diseases, such as atherosclerosis (Hopkins 2013). Depending on its concentration, H_2O_2 induce relaxation or constriction of blood vessels. High concentrations of H_2O_2 have been shown to induce contraction (Gil-Longo and Gonzalez-Vazquez 2005; Silva et al. 2017). In the present study, 1 mM H_2O_2 transiently contracted the aortic rings. The presence of the endothelium did not affect the contractions; similar responses were observed in the E+ and E– groups (Fig. 2B).

The effect of the endothelium on responses to H_2O_2 mainly depends on the type of vasculature and H_2O_2 concentration (Rodriguez-Martinez et al. 1998; Gao and Lee 2005; Garcia-Redondo et al. 2009; Csato et al. 2014). Our findings in the aorta are similar to those of previous studies that demonstrated increased H_2O_2-induced contractile activities in resistance arteries and skeletal muscle arterioles with an intact endothelium (Garcia-Redondo et al. 2009; Csato et al. 2014). The increase in H_2O_2-induced contractions despite the presence of the endothelium indicates impaired endothelial functioning or NO bioavailability (Gao and Lee 2005; Feletou et al. 2011; Vanhoutte 2011; Silva et al. 2017). However, the E+ PMF group exhibited reduced H_2O_2-induced constrictions in the present study, indicating that PMF application affects the modulation of endothelial activity and thereby improves endothelial functioning during oxidative stress.

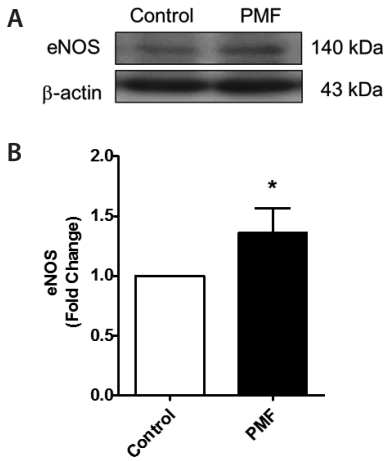

Figure 4. eNOS levels in PMF-applied aortic tissues. **A.** Western blot data of eNOS and β-actin expression levels in the Control and PMF groups. **B.** eNOS protein bands were normalized to β-actin bands. Bar graph represents the mean ± SD, n = 9–10 rats/group. * $p < 0.05$ *vs.* Control group (by Student's t-test).

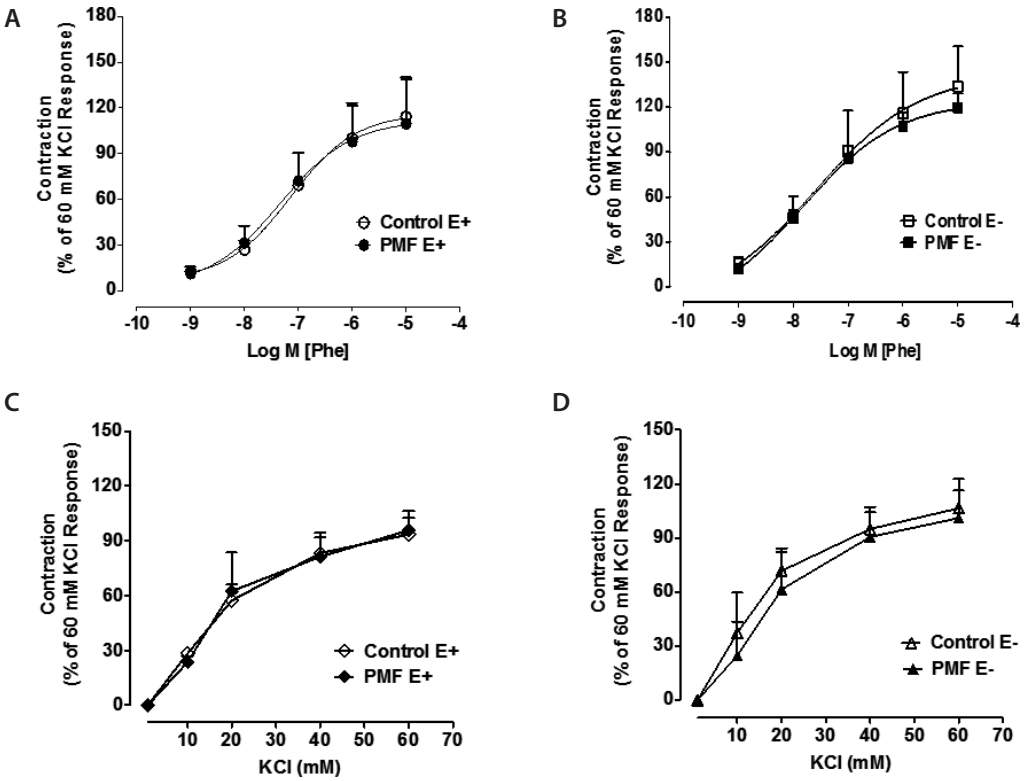

Figure 5. Effect of PMF on Phe- and KCl-induced contractions of E+ and E− aortic tissues**.** Dose-response curve for Phe in E+ rings **(A)** and E− rings **(B)** in control and PMF groups. Concentration-response curve for KCl in E+ rings **(C)** and E− rings **(D)** in Control and PMF groups. Results are shown as the mean ± SD and expressed as the percentage of maximal contraction induced by 60 mM KCl. n = 9–10 rats/group.

Pathologically increased H_2O_2 is related to impaired eNOS function in blood vessels (Maron and Michel 2012). In order to assess the effect of PMF on endothelial functioning, we induced apoptosis with H_2O_2 incubation and observed the impact of PMF on NO regulation. We incubated aortic tissues with 1 mM H_2O_2 for 40 min, the adequate dose and time to induce early apoptosis (Wang et al. 2007; Yan et al. 2017). The data of Bcl-2 expression are also indicative of H_2O_2-induced apoptosis (Fig. 3A and B). During H_2O_2 incubation, we added high-dose KCl to induce contractions with the simplest electrophysiological mechanism (Ratz et al. 2005) (Fig. 3C). We observed significantly reduced vasoconstrictions in the E+ PMF group, indicating the effect of PMF on the endothelium during apoptosis.

Our findings reveal an association between PMF and endothelial functioning. These findings are consistent with those of previous studies, which have shown that PMF application decreases blood pressure in hypertensive rats and increases NO release in HUVECs (Ma et al. 2016). We also observed higher eNOS levels in the E+ PMF group than in the E+ control group, which strongly suggests the interplay between NO and PMF (Fig. 4A and B).

To understand whether the observed effects of PMF are specific to H_2O_2-induced vasoconstrictions, we used Phe or KCl as different signaling pathway activators in blood vessels (Fig. 5). Contractions induced by G protein-coupled receptor activation (Phe) and membrane depolarization (KCl) did not differ significantly in the PMF group, indicates that PMF particularly exerts its effect through H_2O_2-induced vasoconstricting agents.

We investigated the differences in aortic contractions in response to various stimuli after 30 days of *in vivo* PMF application in rats and observed that the endothelial activity was maintained in the PMF group on high-dose H_2O_2 stimulation.

Alterations in the frequencies of weak magnetic fields can change the energy levels of molecules and electrons and may even cause radical and radical pair formation with singlet (S) to triplet (T) state transformations (Steiner and Ulrich 1989; Woodward 2002). Such occurrence in biological systems may alter chemical reaction kinetics and thereby affect the concentrations of radicals, causing the differentiation of biochemical interactions (Barnes and Greenebaum 2015). In addition, the production of H_2O_2, which is another radical, changes magnetic field applications (Usselman et al. 2014). However, under normal physiological conditions and in the presence of magnetic fields, biological systems regulate the formation of these radical pairs with radical scavengers (Katsir and Parola 1998; Barnes and Greenebaum 2015). In each circumstance, the modulation of these scavengers can result in changes in their expression levels or activities (Simko 2007). This modulation may be a basis for the change in endothelium activity on oxidative stress stimulation in the PMF group.

In summary, our findings indicate that exogenous H_2O_2-induced constrictions are modulated by a more active aortic

endothelium on PMF application. This finding suggests an interplay between PMF and the endothelium, as revealed *via* ROS-induced homeostatic disruptions, suggesting the benefits of PMF as an adjuvant to treatments. However, further studies need to be conducted to understand the effects of PMF in detail.

Conflict of interest. The authors have no conflicts of interest to declare

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