Exposure to 50 Hz electric fields reduces stress-induced glucocorticoid levels in BALB/c mice in a kV/m- and duration-dependent manner

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Running head: Anti-stress effects of 50 Hz EF in mice

Abstract

Electric fields (EFs) can reduce elevated levels of stress-related hormones in some organisms. In this study, the endocrine effects of exposure to a 50 Hz EF were investigated in male BALB/c mice. Specifically, plasma glucocorticoid (GC) levels were examined because GC is known to mediate the stress response in mice, including changes induced by immobilization. The mice were exposed to 50 Hz EFs (at 2.5 to 200 kV/m) for 60 min, and they were immobilized for the latter half (30 min). At the end of the exposure period, blood samples were collected and GC levels were estimated by spectrofluorometry. GC levels were not influenced by EFs in absence of immobilization, but they were significantly higher in immobilized mice than in non-immobilized mice ($P < 0.01$). The elevated GC levels induced by immobilization were significantly reduced by exposure to an EF at 10 kV/m ($P < 0.05$), and the effect of EFs at 0 to 10 kV/m on GC levels increased in a kV/m-dependent manner ($P < 0.05$). In contrast, following treatment with EFs at 50 and 200 kV/m, GC levels were higher than those observed at 10 kV/m. To assess the effect of EF treatment duration, mice were also exposed to 50 Hz EFs (10 kV/m) for 6, 20, or 60 min. The immobilization-induced increase in GC levels was significantly suppressed by EF exposure for 20 and 60 min. Therefore, our results demonstrate that extremely low-frequency EFs alter the stress response of mice in a kV/m- and duration-dependent manner.

Key words: electric field, stress, dose-dependent, glucocorticoid

Introduction

In recent decades, the distribution and use of electricity has increased rapidly not only in developed countries but also in developing nations. This widespread use of electricity in domestic and industrial settings has resulted in increased human exposure to electric fields (EFs); therefore, it is important to understand the biological effects of EFs, particularly at 50 and 60 Hz power-line frequencies [Hjeresen et al., 1980; Jaffe et al., 1980; Marino et al., 1980; Jaffe et al., 1981]. Induced electric current in the body and the perception of EFs on the surface of the skin can trigger cellular and humoral responses in certain organisms [Weigel and Lundstrom, 1987; Weigel et al., 1987; Kato et al., 1989; Romo et al., 1998, 2000, 2002]. Additionally, mechanical vibrations on the surface of the skin at 5–50 Hz, which are similar to the perception of EFs, activate neurons in the primary somatosensory cortex that correspond to the site of stimulation [Romo et al., 1998]. Some studies have shown that EFs may have therapeutic effects; for example, EFs have been used to treat bone diseases and stimulate growth via increase of alkaline phosphatase activity and calcium incorporation, compared with controls [Bassett et al., 1981; Takano-Yamamoto et al., 1992]. Indeed, the United States Food and Drug Administration first approved devices that apply electrical energy to the skin surface, resulting in induced electric currents that mediate desired therapeutic effects, in 1978. Furthermore, in 1972, the Ministry of Health and Welfare in Japan approved the manufacture of physical therapy apparatus that use 50 or 60 Hz EFs to alleviate pain related to shoulder stiffness, insomnia, chronic constipation, and headaches [Hara, 1961]. Previously, we described that EFs can modulate certain biological systems, such as the endocrine and immune systems [Harakawa et al., 2004a; Hori et al., 2005], in addition to cell signaling pathways [Harakawa et al., 2004b]. In addition, in vitro studies have suggested that exogenous EFs can induce changes in intracellular calcium ion concentrations and protein synthesis [McLeod et al., 1987; Cho et al., 1999]. Although the in vitro and in vivo effects of high-frequency EFs and magnetic fields have been investigated [Kim et al., 2013; Romanenko et al., 2014], analyses of

the in vivo effects of power line-frequency EFs have not been analyzed. In order to conduct such analyses, it will first be necessary to establish experimental methods to assess the quantitative and qualitative effects of exogenous EFs. We previously reported that EF exposure reduces elevated levels of plasma adrenocorticotropic hormone (ACTH) induced by immobilization in Wistar rats [Harakawa et al., 2004a]. In contrast, when mice were exposed to 50 Hz 10 kV/m, the serum corticosterone concentration was higher than that in the controls [de Bruyn, 1994.]. The corticosterone, which is a main glucocorticoid (GC), is produced by the adrenal gland, and changes in corticosterone are generally interpreted to indicate stress. Thus, the authors concluded that the EF was a stressor. However, another former study noted that reduced blood glucocorticoid concentrations were observed in rats exposed to a 60 Hz 15 kV/m [Marino et al., 1977]. Furthermore, exposure to up to 60 Hz 50 kV/m induced reductions in plasma glucocorticoid concentrations, but only at the beginning of the exposure period [Hackman et al., 1981]. Others found no increase in glucocorticoid levels after 30–120 days of field exposure in adult male rats [Free, 1981]. Glucocorticoid, a hormone that generally mediates the stress response, is released from the adrenal gland by ACTH stimulation. Therefore, in this study, we further investigated the effects of EF exposure in mice; specifically, we evaluated the kV/m- and duration-dependent effects of EF on elevated GC levels induced by short-term immobilization in mice.

Materials and Methods

Animals

Eight-week-old male BALB/c mice were purchased from CLEA Japan (Tokyo, Japan) and maintained in a pathogen-free environment at 24 °C ± 1 °C, 50% ± 10% humidity, with daily artificial illumination (12 h light/dark cycle with lights on from 7:00 am to 7:00 pm). The animals had free access to standard laboratory chow (CE-2; CLEA Japan) and water except for the period during EF exposure and immobilization. All animal experiments described in this article were conducted in

accordance with the Guiding Principles for the Care and Use of Research Animals Promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 25–86).

EF exposure system

The EF exposure system comprised three major parts: a high-voltage transformer unit (A30; maximum output voltage, 30 kV; Hakuju, Tokyo, Japan), a constant-voltage unit (CVFT1-200H; Tokyo Seiden, Tokyo, Japan) to avoid unexpected interference from electrical noise originating from the commercial power supply, and EF exposure cages [Harakawa et al., 2005, 2008; Hori et al., 2012]. The exposure cages comprised a cylindrical plastic cage (diameter, 200 mm; height, 100 mm) and two stainless steel electrodes (1,000 × 600 mm) that were placed over and under the cylindrical cage (Fig. 1A). The cylindrical cage had slits (length, 100 mm; width, 5 mm) all around at intervals of 5 mm (Fig. 1B) to prevent smudges (from feces or saliva) from disturbing the formation of a stable EF. To generate the EF (50 Hz, 2.5 to 200 kV/m root mean square) in the cage, an electrical current of 50 Hz, 0.25–20 kV was applied to the upper electrode, whereas the lower electrode was grounded. To measure the field intensity and verify the system's operation, an optical fiber voltmeter, which measures EF intensity by Pockels effect, and an electro optic voltage sensor attached with two-cored $Bi_{12}SiO_{20}$ -fiber (FOVM 03; Sumitomo Electric, Osaka, Japan) and a digital multimeter (Fluke 87; Fluke, Everett, WA) were used. EF intensity on arbitrary 273 points (21 x 13) of a cage floor was measured. The EF intensities of 2.5 to 200 kV/m applied to the cage in which the mouse was kept had a margin of error of ± 1%. The temperature within the cylindrical cage did not change during the EF- or sham-exposure period.

Immobilization stress

Each mouse was restrained within a 50-mL centrifuge tube and laid on the lower electrode (Fig. 1C)

[Inoue et al., 2009].

kV/m dependency of EF effect

To examine the effect of EF alone, i.e., without immobilization stress, on the plasma GC level, eight-week-old male BALB/c mice were divided into three groups (n = 5–6 per treatment group): a control group [Stress/EF (−/−)] and two EF-alone groups [Stress/EF (−/50 Hz, 10 kV/m for 60 min) and Stress/EF (−/50 Hz, 150 kV/m for 60 min)]. To investigate the kV/m dependency of the EF effect, eight-week-old male BALB/c mice were divided into seven treatment groups, with n = 8 per treatment group (Fig. 2): a control group [Stress/EF (−/−)], an immobilization-alone group [Stress/EF (+/−)], and immobilization-EF co-treated groups [Stress/EF (+/50 Hz, 2.5 kV/m for 60 min), Stress/EF (+/50 Hz, 5 kV/m for 60 min), Stress/EF (+/50 Hz, 10 kV/m for 60 min), Stress/EF (+/50 Hz, 50 kV/m for 60 min), and Stress/EF (+/50 Hz, 200 kV/m for 60 min)]. Mice in the EF-treated groups were always exposed to 50 Hz EF for 60 min, and those in the co-treated groups were immobilized in the second half (30 min) of the EF exposure period. Mice in the control group, Stress/EF (−/−), were handled in a similar manner except that the EF condition was 0 V/m. That is, they were housed within the EF cage for 60 min.

Dependence of EF effect on duration of exposure

To assess the dependence of the EF effect on the duration of exposure, eight-week-old male BALB/c mice were divided into five treatment groups (Fig. 3), with n = 8 per group: control [Stress/EF (−/−)], immobilization-alone [Stress/EF (+/−)], and co-treated groups [Stress/EF (+/50 Hz, 10 kV/m for 6 min), Stress/EF (+/50 Hz, 10 kV/m for 20 min), and Stress/EF (+/50 Hz, 10 kV/m for 60 min)]. All the mice spent 60 min in the EF exposure cage. Stress treatments were identical to previous experiments (30 min immobilization in the second half of a 60 min test period), but the EF-treated mice were exposed to 50 Hz EF for 6, 20, or 60 min.

Derivatization and fluorimetric assay of plasma GC levels

Immediately after the EF treatments, 800 μL of blood was collected from each mouse under anesthesia (3% isoflurane; Mylan, Tokyo, Japan). Blood collection was performed between 10:00 am and 12:00 pm. The blood samples were centrifuged at 1,500 ×*g* for 10 min at 4 °C, and the plasma was collected and stored at −80 °C until further use.

To derivatize GC, plasma (200 μL) was first mixed with 900 μL of isooctane (2,2,4-trimethylpentane; Wako, Osaka, Japan). The sample was then vortexed and centrifuged at 380 ×*g* for 5 min at room temperature. The upper layer (supernatant) was discarded and 900 μL of chloroform (Wako) was added to the lower layer. Subsequently, the sample was vortexed and centrifuged at 380 ×*g* for 5 min at room temperature. The supernatant and white membranous layers were removed, and the lower layer was retained for further analysis. The lower layer (800 μL) was transferred to a new tube, mixed with 320 μL of a solution containing 65% concentrated sulfuric acid (Wako) and 35% ethanol (Wako), and then vortexed. The solution was incubated in the dark for 3.5 h, and the fluorescence intensity of the sample was measured at 519 nm with excitation at 475 nm using a spectrofluorophotometer (RF-5300PC; Shimadzu, Kyoto, Japan).

Statistical analysis

The results are expressed as the mean ± standard deviation. Differences among all groups were evaluated by one-way analysis of variance and those between two groups were evaluated by Tukey's multiple comparison test. Significance was defined as $P < 0.05$. All statistical analyses were conducted using Prism Version 6 (GraphPad Software, La Jolla, CA).

Results

Dose-dependent effect of EF exposure

There was no significant difference in the plasma GC levels observed in mice that were treated with

EFs but not immobilization (50 Hz, 10 or 150 kV/m for 60 min) and mice in the control group (Fig 4A). However, plasma GC levels were significantly higher in mice treated with immobilization stress alone [Stress/EF (+/−)] than that in control group mice [Stress/EF (−/−)] ($P < 0.01$; Fig. 4B). GC levels in the co-treated groups [Stress/EF (+/+)] were lower than those in the immobilization-alone group [Stress/EF (+/−)], and this trend appeared to be kV/m-dependent in the range from 2.5 to 10 kV/m ($P < 0.05$). Additionally, GC levels were higher in mice treated with EFs at 50 and 200 kV/m than in those treated with EFs at 10 kV/m. When the GC levels were plotted against the voltage, the resulting graph had an inverted bell shape (Fig. 4B).

Plasma GC levels in mice that were immobilized and exposed to an EF (10 kV/m) for 6 min were not significantly different from those in mice that were immobilized but not treated with EF; however, GC levels were significantly lower in immobilized mice treated with EF for 20 and 60 min (Fig. 5).

Discussion

In this study, the effect of a 50 Hz EF on immobilization-induced changes in plasma GC levels was examined in BALB/c mice. The mice were stressed by immobilization within a centrifuge tube for 30 min. Plasma GC levels increased by approximately 5-fold after immobilization, suggesting that the immobilization procedure affected the pituitary-adrenocortical axis and the sympathetic-adrenomedullary system, which manifested as stress [Kvetnansky et al., 1979; Sudo and Miki, 1993; Yamada et al., 1996; Arakawa et al., 1997]. Our results suggest that EF exposure suppressed the immobilization-induced increase in GC levels in mice, indicating that EF has an anti-stress and/or suppressive effect on GC secretion. These data are consistent with the results of our previous study, which showed that immobilization-induced elevation of stress hormone levels could be suppressed in rats by a 50 Hz EF at 17.5 kV/m [Harakawa et al., 2004a]. To the best of our knowledge, this is the first in vivo study to demonstrate that extremely low-frequency EFs can alter

stress responses in a kV/m-dependent and treatment-duration-dependent manner. Additionally, our data indicated that the effect of EF exposure was attenuated at 50 and 200 kV/m. It is conceivable that the anti-stress effect of EFs occurs at optimum conditions of <50 kV/m, above which the effect is attenuated and/or unexpected factors would interfere the effect of EFs. For example, the mechanical vibration of electrodes to form EF would be turned up due to intensity of applied signal. Unexpected vibrations on the exposure system were measured by with a portable digital vibration meter. Vibration (m/s^2) at the center of the upper electrode was detected when 5 kV (50 kV/m) and above was applied to the lower electrode (data not shown). We supposed that frequency of the detected vibration might be a 50 Hz. In the primitive test, the intensity of vibration was found a tendency to be positively correlated with the magnitude of the applied voltage. Therefore, it is considered that the data on GC levels recorded at 50 kV/m and above might be affected by both EF exposure and vibration. However, in order to solve this hypothesis, one control group is needed, that is pure vibration (50 Hz) + stress group without EF. These aspects need to be examined in future studies.

The experimental system described in this report could be applied to other studies to investigate the effect of EFs on mice and other organisms. However, the animals' sensitivity to stress is an important factor that should be considered before conducting experiments to detect the effects of EFs on stress hormone levels. For example, immobilization-induced changes in plasma GC levels were ambiguous in both the ICR and C57BL/6J mice (data not shown).

In conclusion, we found that exposure to EFs can suppress stress-induced GC secretion in a dose-dependent manner in BALB/c mice. Furthermore, we have developed an experimental model to assess the effect of EFs in vivo and demonstrated that 50 Hz EFs can modulate the endocrine system in stressed mice.

Acknowledgments

The authors greatly thank Mr. Fuyuki Doge, Hakuju Institute for Health Science and Dr. Masahiro Tsuchiya, National Institute of Information and Communications Technology, for their advices and constructive criticisms on the descriptions regarding electrical engineering during the preparation of this manuscript. We would like to thank Editage (www.editage.jp) for English language editing.

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Figure legends

Figure 1. Electric field (EF) exposure system. A: Voltage generator and electrodes. B: A mouse within an exposure cage. C: A restrained mouse.

Figure 2. Experimental design used to analyze the kV/m dependency of the EF effects. The mice were divided into the following groups (n = 8 per treatment group): control, Stress/EF (−/−); immobilization-alone, Stress/EF (+/−); co-treated groups, all exposed at 50 Hz for 60 min, Stress/EF (+/2.5 kV/m), (+/5 kV/m), (+/10 kV/m), (+/50 kV/m), and (+/200 kV/m). Co-treated mice were exposed to 50 Hz EF for 60 min, and were immobilized in the second half (30 min) of the EF exposure period. In the control group, Stress/EF (−/−), mice were handled in an identical manner except that the EF condition was 0 V/m and they were not immobilized.

Figure 3. Experimental design used to analyze the dependency of the EF effects on exposure duration. The mice were divided into the following groups (n = 8 per treatment group): control, Stress/EF (−/−); immobilization-alone, Stress/EF (+/−); and co-treated groups, Stress/EF (+/50 Hz, 10 kV/m for 6 min), Stress/EF (+/50 Hz, 10 kV/m for 20 min), and Stress/EF (+/50 Hz, 10 kV/m for 60 min). All mice spent 60 min in the EF exposure cage. Mice in the stress groups were immobilized in the second half (30 min) of the 60 min test period.

Figure 4. A: Effect of EFs on plasma glucocorticoid (GC) levels. There were no significant differences. B: Effect of immobilization on plasma GC levels and effect of EFs on immobilization-induced increase in plasma GC levels. GC levels were lower in the co-treated group Stress/EF (+/+) than that in the immobilization-alone group Stress/EF (+/-), and kV/m dependency was observed in the range from 2.5 to 10 kV/m ($P < 0.05$). GC levels were higher in mice exposed to EFs at 50 and 200 kV/m than in mice exposed to an EF at 10 kV/m.

Figure 5. Effects of the duration of EF exposure on immobilization-induced increase in plasma GC

levels. Plasma GC levels in mice that were immobilized and exposed to an EF (10 kV/m) for 6 min were not significantly different from those in mice that were immobilized but not treated with EF; however, GC levels were significantly lower in mice that were immobilized and treated with EF for 20 and 60 min.

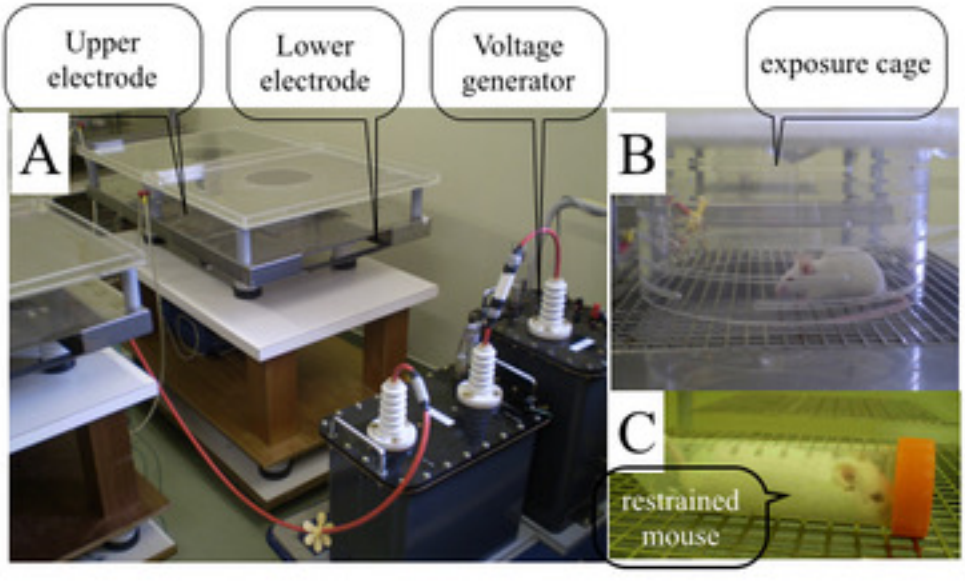
Upper electrode
Lower electrode
Voltage generator
exposure cage
A
B
C
restrained mouse

Figure 1

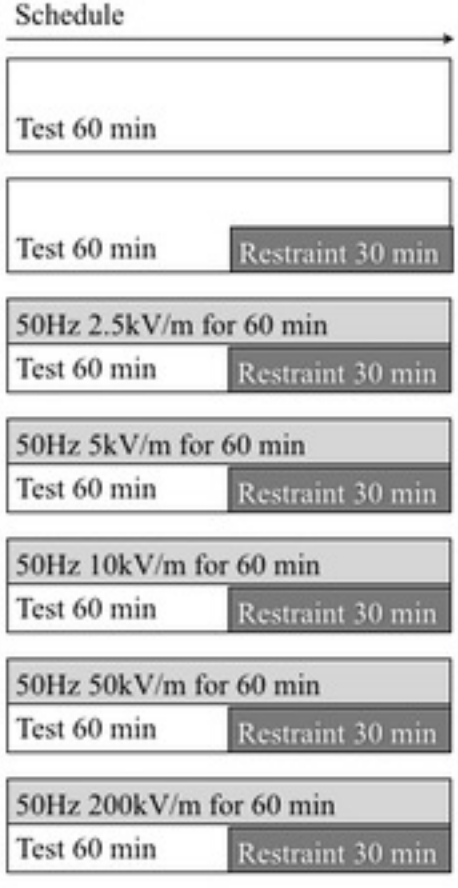
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Figure 2

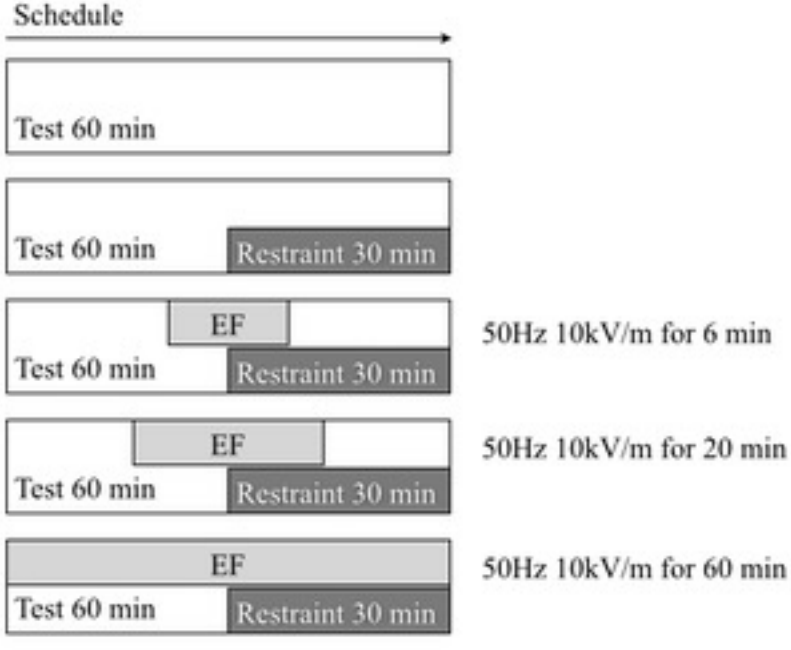
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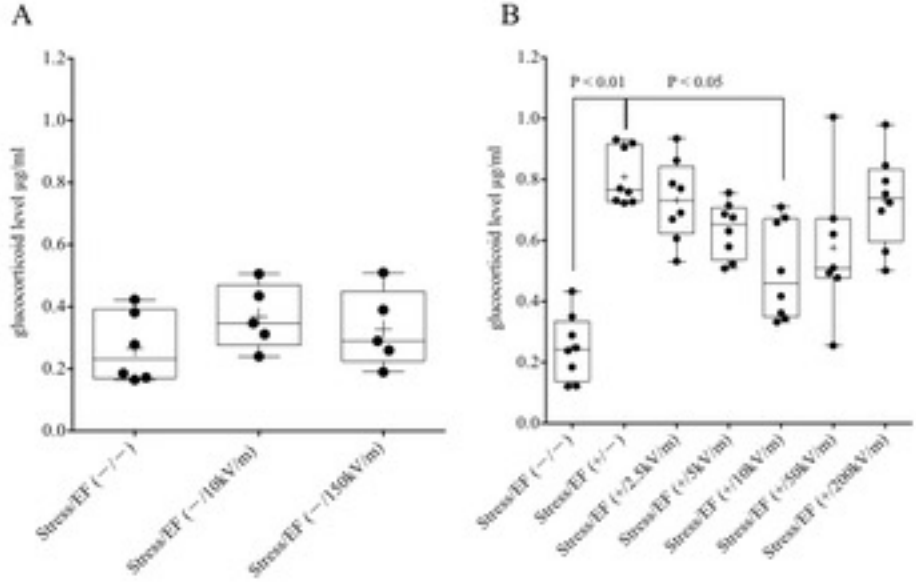
Figure 3


A
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1.0
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Stress/EF (−/−)
Stress/EF (−/10kV/m)
Stress/EF (−/15kV/m)
Stress/EF (+/−)
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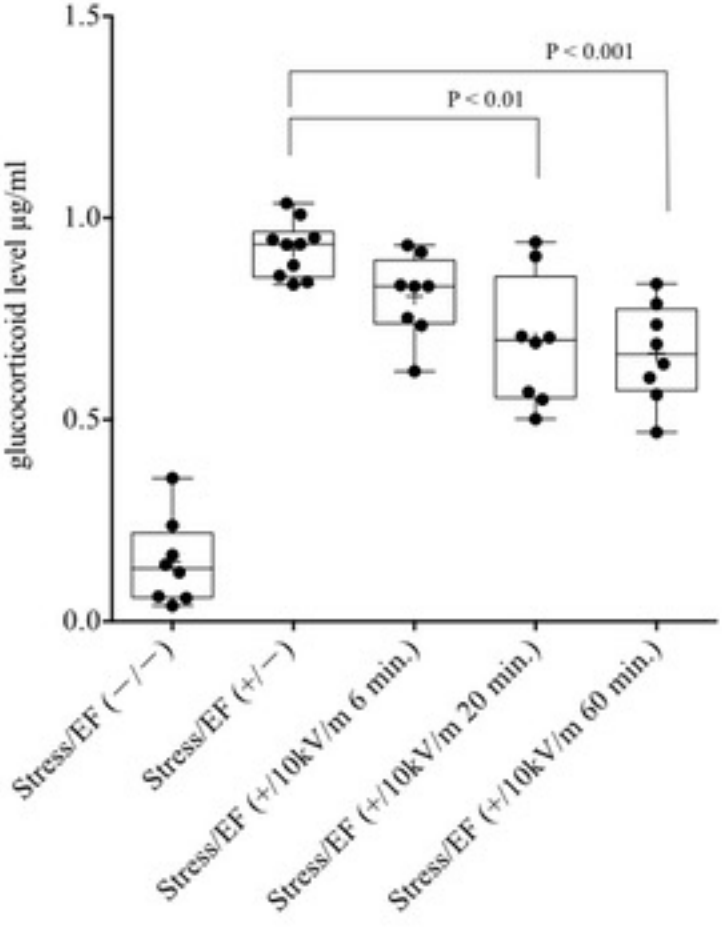
Figure 4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Stress/EF (+/10kV/m 20 min.)
Stress/EF (+/10kV/m 60 min.)

Figure 5