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Time-dependent changes in the suppressive effect of electric field exposure on the immobilization-induced plasma glucocorticoid increase in mice

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

Conflict of interest: None

Abstract

We recently reported that increased glucocorticoid (GC) levels in immobilized mice were suppressed by exposure to a 50-Hz electric field (EF) in kV/m-dependent and exposure duration-dependent manners. Here, we characterized the time-dependent changes in the effect of EF exposure in immobilized mice. Using control, EF-alone, immobilization-alone, and co-treated groups, plasma GC levels and blood properties were first measured (0-60 min) to observe changes induced by each treatment and measured again (60-120 min) to assess the recovery from each treatment. The 50-Hz, 10-kV/m EF was formed in a parallel plate electrode. Co-treated mice were exposed to the EF for 60 min for the first measurement and were immobilized for the second half (30-60 min) of the EF exposure period. Plasma GC levels did not change significantly over time in the control and EF-alone groups. The GC levels in the immobilization-alone and co-treated groups increased after immobilization, peaking 30 min after the start of immobilization and then decreasing gradually; however, the GC peak was lower in the co-treated group than in the immobilization-alone group ($P < 0.05$ at 50 and $P < 0.001$ at 60 min). The red blood cell counts, hemoglobin levels, and hematocrit values increased after immobilization but were not affected by the EF. Our findings indicate that the EF did not shift the peak of the time-dependent increase in plasma GC levels in immobilized mice but simply reduced it.

Key words: stress; endocrine response; electrical stimulation

Introduction

Given the widespread use of electricity in domestic and industrial settings, it is important to understand the biological effects of electric fields (EFs), particularly at the 50- and 60-Hz frequencies of power lines. Although World Health Organization (2008) have indicated that exposure to ELF electric fields also induces a surface electric charge which can lead to perceptible, but non-hazardous effects, including microshocks, only a limited number of studies have been performed in this respect to date, and therefore there is insufficient evidence to reach a clear understanding of the potential hazardous or beneficial effects of EF [Hjeresen et al., 1980; Jaffe et al., 1980; Marino et al., 1980; Jaffe et al., 1981]. The induction of electric currents in the body and the perception of EFs on the skin surface 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]. Some studies have shown that EFs may have therapeutic effects, e.g., EFs have been used to treat bone diseases and to stimulate bone growth by increasing alkaline phosphatase activity and calcium incorporation [Bassett et al., 1981; Takano-Yamamoto et al., 1992]. Indeed, in 1979, the United States Food and Drug Administration approved the use of devices that apply electrical energy to the skin surface, resulting in induced electric currents that mediate favorable therapeutic effects [FDA, 2006]. In 1972, the Ministry of Health and Welfare in Japan approved the manufacture of a physical therapy apparatus that use AC 50 or 60-Hz EFs to alleviate the pain related to shoulder stiffness, insomnia, chronic constipation, and headaches. Previously, we reported that EFs can modulate certain biological systems, such as the endocrine and immune systems [Harakawa et al., 2004b; Hori et al., 2005; Kariya et al., 2006], and cell signaling pathways [Harakawa et al., 2004a]. In addition, the results from *in vitro* studies have suggested that an exogenous EF can alter intracellular calcium ion concentrations and protein synthesis [McLeod et al., 1987; Cho et al., 1999].

In order to elucidate the biological effects of power line-frequency EFs, it is necessary to establish experimental methods for assessing the quantitative and qualitative effects of exogenous EFs. We previously reported that EF exposure reduced the elevated levels of plasma adrenocorticotropic hormone (ACTH) induced by immobilization in Wistar rats [Harakawa et al., 2004b]. In contrast, when mice were exposed to 50 Hz at 10 kV/m, their serum corticosterone concentrations were higher than those of the controls [de Bruyn and de Jager, 1994]. Corticosterone, which is the main glucocorticoid (GC), is produced by the adrenal gland, and changes in corticosterone are generally indicators of stress. Thus, de Bruyn and de Jager (1994) concluded that the EF was a stressor to the rats. However, another previous study reported reduced blood GC levels in rats

exposed to a 60-Hz, 15-kV/m EF compared to control rats [Marino et al., 1977]. Furthermore, exposure to up to a 60-Hz, 50-kV/m EF induced a reduction in plasma GC concentrations, but only at the beginning of the exposure period [Hackman and Graves, 1981]. Another study reported no increase in GC levels in adult male rats after 30–120 d of EF exposure [Free et al., 1981]. GCs are involved in mediating the stress response and are released from the adrenal gland upon ACTH stimulation. While, some previous studies reported that 60 Hz magnetic field might induce a secretion of catecholamine from adrenal grand by Graviso (2002, 2003), controversial issue was remained on the effect of fields in the hormone release or secretion.

We recently found that an immobilization-induced increase in the GC level was reduced in male BALB/c mice after exposure to a 50-Hz EF in a kV/m-dependent manner and exposure duration-dependent manner [Hori et al., 2015]. In this study, we further assessed the time-dependent changes in the effect of EF exposure on the endocrine response to immobilization treatment in mice.

Materials and Methods

Animals

Eight-week-old male BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan) and maintained in a pathogen-free environment at 24 ± 1 °C, with 50 ± 10% humidity and daily artificial illumination (12-h light/dark cycle with lights on from 7:00 to 19:00). The animals had free access to standard laboratory chow (CE-2; CLEA, Tokyo, Japan) and water, except for the period during EF exposure and immobilization. All animal experiments described in this study 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 Obihiro University of Agriculture and Veterinary Medicine (Permit number 25–86).

EF exposure system

The EF exposure system consisted of 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; Hori et al., 2005; Harakawa et al., 2008; Hori et al., 2015]. The exposure system comprised a cylindrical plastic cage (diameter, 200 mm; height, 50 - 200 mm) and two stainless steel electrodes (1,000 × 600 mm) that were placed over and under the cylindrical cage (Fig. 1A:

outward, 1B: electrodes and cylindrical cage, 1C: mouse in cylindrical cage, 1D: immobilized mouse). 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 in the cage, 50 Hz, 1 kV was applied to the upper or lower electrode, and the counterpart electrode was grounded. A separate cage and tube were used for each animal; the cages and tubes were washed with a neutral detergent and completely dried before reuse. To measure the field intensity and verify the system's operation, an optical fiber voltmeter, which measures EF intensity by the Pockels effect; an electro-optic voltage sensor attached with two-cored Bi12SiO20 -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 intensity of 10 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. The humidity was kept from 45 to 55%.

Time-dependent changes in plasma GC levels and blood properties

To assess the time-dependent changes in the effect of EF exposure in immobilized mice, a 120-min exposure was divided into two periods, a first half (60 min) and second half (60 min) (Fig. 3). During the first half, changes in GC levels and blood properties were assessed in mice treated with either stress or an EF using the following groups: a control group [Stress/EF (−/−)], an EF-alone group [Stress/EF (−/50 Hz, 10 kV/m for 60 min)], an immobilization-alone group [Stress/EF (+/−)], and an immobilization-EF co-treated group [Stress/EF (+/50 Hz, 10 kV/m for 60 min). The second half was used to assess the recovery patterns from each treatment using the following groups: [post-Stress/EF (−/−)], [post-Stress/EF (−/50 Hz, 10 kV/m for 60 min)], [post-Stress/EF (+/−)], and [post-Stress/EF (+/50 Hz, 10 kV/m for 60 min)]. The control group and EF-alone group contained six sub-groups to allow blood sampling every 10 min after the experiment started (n = 8 per sub-group). The immobilization-alone group and immobilization-EF co-treated group contained three sub-groups to allow blood sampling every 10 min after initiation of immobilization (n = 8 per sub-group). All procedures were performed between 9:00 and 12:00 to avoid influences of circadian changes in GC secretion.

GC extraction and fluorometric assay for 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 and 12:30. Immediately after blood collection, 10 μL of each blood sample was used to measure blood properties using a CellTac blood

analyzer (Nihon Kohden, Tokyo, Japan), and the remainder of each sample was centrifuged at 1500 ×*g* for 10 min at 4 °C, after which the plasma was collected and stored at −80 °C until further use.

To extract 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 two-way analysis of variance and those between two groups were evaluated by Tukey's multiple comparison test. Interactions between time and plasma level of GC were assessed by Pearson correlation analysis and slope regression analysis. Significance was defined as $P < 0.05$. All statistical analyses were conducted using Prism Version 6 (GraphPad Software, La Jolla, CA).

Results

The circadian changes in GC levels were first assessed, and the plasma GC level in the nine sub-groups sampled from 9:00 to 15:00 showed an incremental change. These changes were not significant between 9:00 and 12:00 but were significant between 9:00 and 15:00 ($P < 0.005$, correlation analysis), indicating a circadian rhythm for GC (Fig. 4).

For the first half test (0-60 min), there were no significant time-dependent changes in the plasma GC levels in mice in the control group and EF-alone group according to a correlation analysis (Fig. 5A). However, the plasma GC levels in mice in the immobilization-alone group and immobilization-EF co-treated group showed increasing trends 30 min after the start of immobilization, and the increases in the immobilization-alone group were significant compared to the control group ($P < 0.0001$). At the end of first half, there were no differences in the GC level of each group between the control and EF-alone groups; in addition, those of the stress alone

group were higher than those of the control group, and those of the co-treatment group were lower than those of the stress alone group (Fig. 5A and C).

The second half test (60-120 min) assessed the time-dependent changes in the plasma GC levels after each treatment was finished (recovery period), and the GC levels in the control and EF-alone groups did not exhibit an increasing or decreasing trend (Fig. 5B). However, the plasma GC levels in mice in the immobilization-alone group ($P < 0.005$) and immobilization-EF co-treated group ($P < 0.05$) decreased over the 60-min period after the end of immobilization or co-treatment.

White blood cell counts did not show any differences among groups (Fig. 6). Red blood cell count, hemoglobin level, and hematocrit (%) increased after the immobilization treatment and then recovered gradually over the 60 min after the end of immobilization. The increases peaked 30 min after the start of immobilization and then showed a downward trend, with no differences observed among groups.

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 subjected to stress by immobilization within a centrifuge tube for 30 min. Plasma GC levels increased by up to approximately 2-fold after immobilization, indicating that the immobilization procedure affected the pituitary-adrenocortical axis of the endocrine system and the sympathetic-adrenomedullary system, manifesting as stress [Kvetnansky et al., 1979; Sudo and Miki, 1993; Yamada et al., 1996; Arakawa et al., 1997]. In this study, EF exposure suppressed the increase in GC levels in immobilized mice, indicating that EF exposure has an anti-stress and/or suppressive effect on GC secretion. These data are consistent with the results of our previous studies, which revealed that the increase in the levels of an immobilization-induced stress hormone is suppressed by a 50-Hz EF at 17.5 kV/m in rats [Harakawa et al., 2004b] and that the immobilization-induced GC increase is suppressed by a 50-Hz EF at 10 kV/m in mice [Hori et al., 2015]. The EF applied in this study was 50 Hz and 1 kV/100 mm (10 kV/m) for 60 min, which was selected because our previous study showed that EFs over 5 kV/100 mm (50 kV/m) intensity generate some vibration and/or noise and because the data on GC levels recorded at 50 kV/m and above might be affected by both EF exposure and vibration [Hori et al., 2015].

The results indicated that a 50-Hz, 1-kV/100 mm (10 kV/m) EF for 60 min induced endocrine changes in the immobilized BALB/c mice. Therefore, the experimental system described in this report may be applied in future

studies to investigate the effect of EFs on organisms.

The results in the first half indicated that the plasma GC levels in the immobilization-alone group increased after immobilization and were higher than those in both the control and EF-alone groups ($P < 0.005$ at 50 and 60 min). The levels in the co-treated mice also increased but were lower than those of the immobilization-alone group ($P < 0.005$ at 50 and 60 min). Taking the data from the first and second halves together, the levels in the immobilization-alone and co-treated groups peaked at 30 min after stress initiation and then decreased gradually. We conclude that the EF did not shift the peak of the immobilization-induced changes in the plasma GC level but simply reduced it. Thus, the time-dependent change in the anti-stress effect of an EF might be caused by the suppression of both the velocity and amount of GC released as a stress response.

GC levels in the present study exhibited circadian changes, seen as a gradual increase, but because the difference between 9:00 and 12:00 was relatively small compared with the differences between the control and immobilization-alone groups or between the immobilization-alone and co-treated groups, these circadian changes could be ignored in the four groups considering the effect of the EF on the GC levels.

Red blood cell count, hemoglobin level, and hematocrit increased after the stress treatment, but they were not affected by the EF. These results were reproducible, but it is not clear why the changes occurred immediately after immobilization started. We speculate that the increase in these blood properties was the result of water deprivation, which caused stress-induced vasoconstriction, but further studies are needed to verify this hypothesis. Furthermore, an EF should have some impact on acute stress-related changes in the endocrine system, but this impact may not affect these blood properties.

In conclusion, our findings demonstrate that an EF reduces immobilization-induced changes in the endocrine system related with the stress response and that the EF does not shift the peak of the time-dependent change but simply reduces it.

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Figure legends

Fig. 1. Electric field (EF) exposure system. A: Voltage generator and electrodes. B: Parallel plate electrodes. C: A mouse in an exposure cage. D: A mouse restrained in a 50-mL centrifuge tube.

Fig. 2. Experimental design used to assess the circadian change in the plasma GC level. Mice were divided into seven groups for blood sampling every 60 min from 9:00 to 15:00 (n = 8 per group).

Fig. 3. Experimental design used to assess the effect of the EF. The first half assessed the changes in GC levels and blood properties while the mice were treated with either stress or an EF using the following groups: a control group [Stress/EF (−/−)], an EF-alone group [Stress/EF (−/50 Hz, 10 kV/m for 60 min)], an immobilization-alone group [Stress/EF (+/−)], and an immobilization-EF co-treated group [Stress/EF (+/50 Hz, 10 kV/m for 60 min). The second half assessed the recovery pattern for each treatment: [post-Stress/EF (−/−)], [post-Stress/EF (−/50 Hz, 10 kV/m for 60 min)], [post-Stress/EF (+/−)], and [post-Stress/EF (+/50 Hz, 10 kV/m for 60 min)] (Fig. 2). The control group and EF-alone group included six sub-groups for sampling blood every 10 min after the experiment started (n = 8 per sub-group). The immobilization-alone group and immobilization-EF co-treated group included three sub-groups for sampling blood every 10 min after initiation of immobilization (n = 8 per sub-group).

Fig. 4. Circadian changes in the plasma GC level from 9:00 to 15:00.

Fig. 5. Effect of immobilization on the plasma GC level and effect of the EF on the immobilization-induced increase in plasma GC.

Fig. 6. Effect of immobilization and the EF on the immobilization-induced increase in blood properties.

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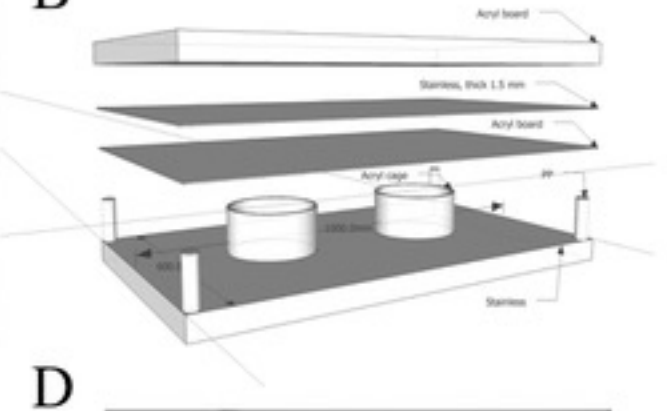
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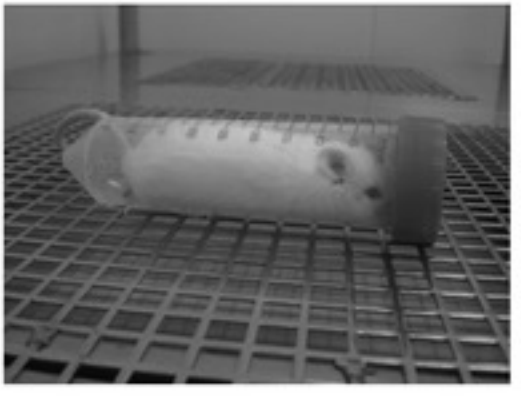

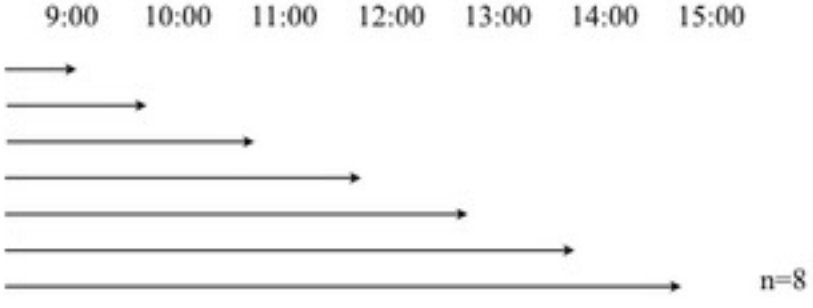
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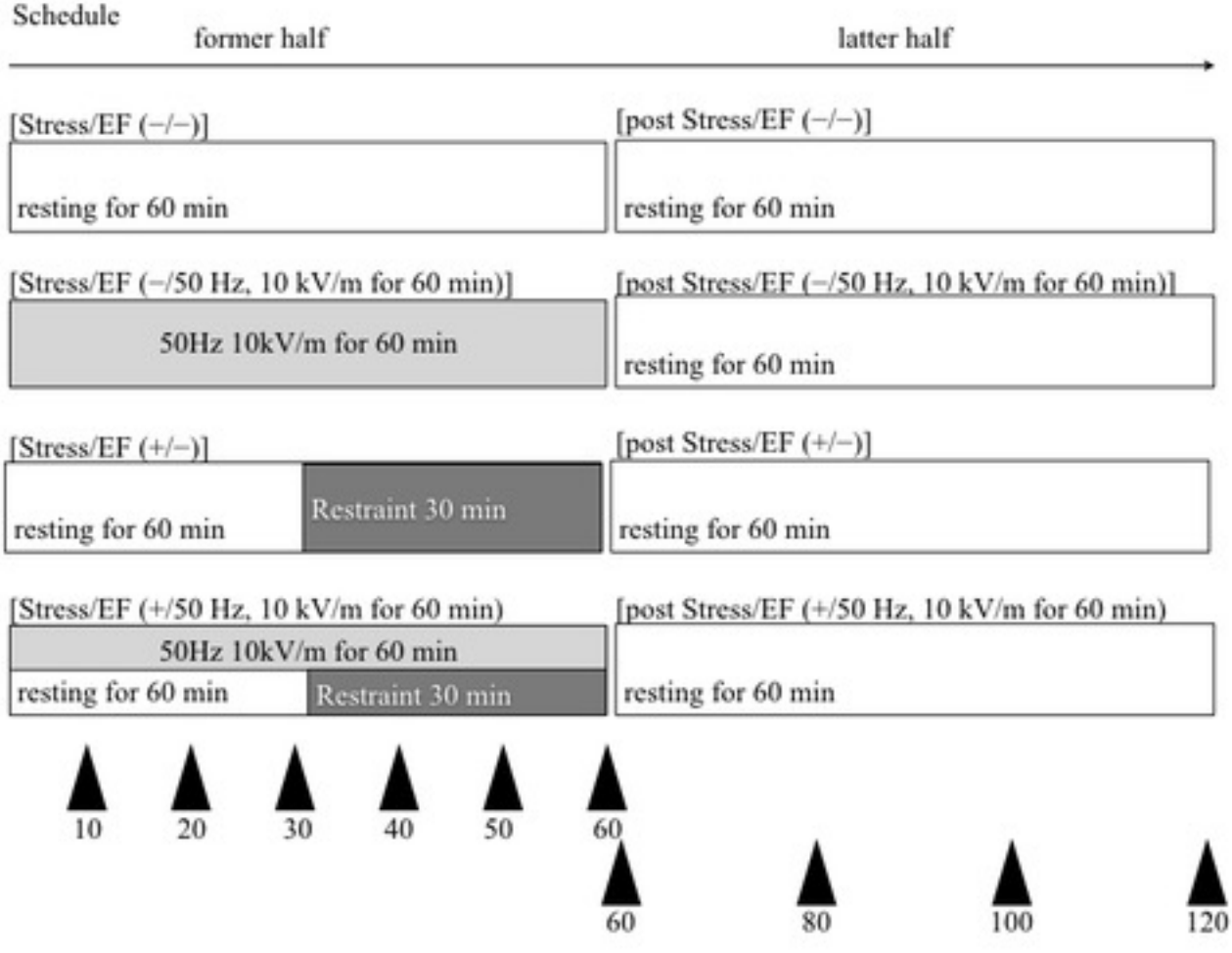
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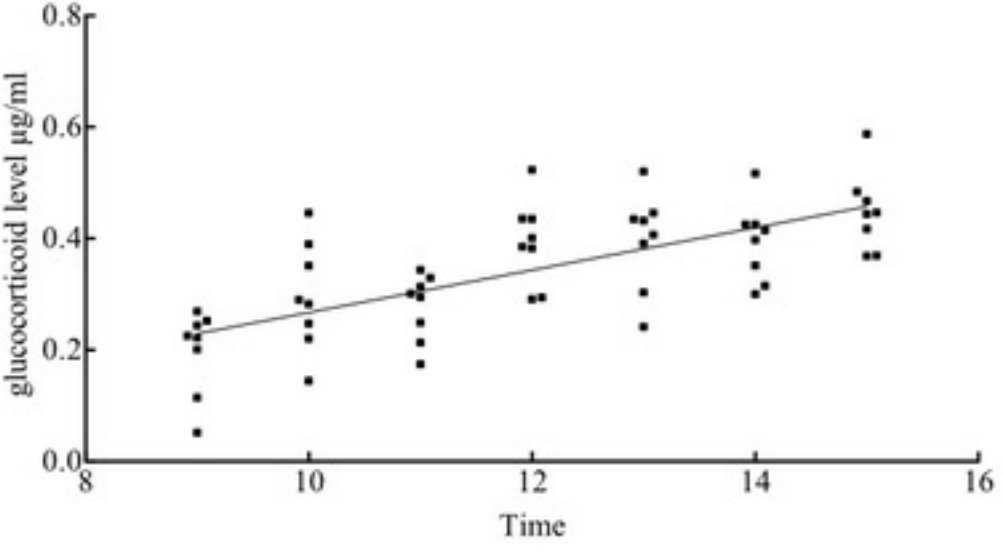
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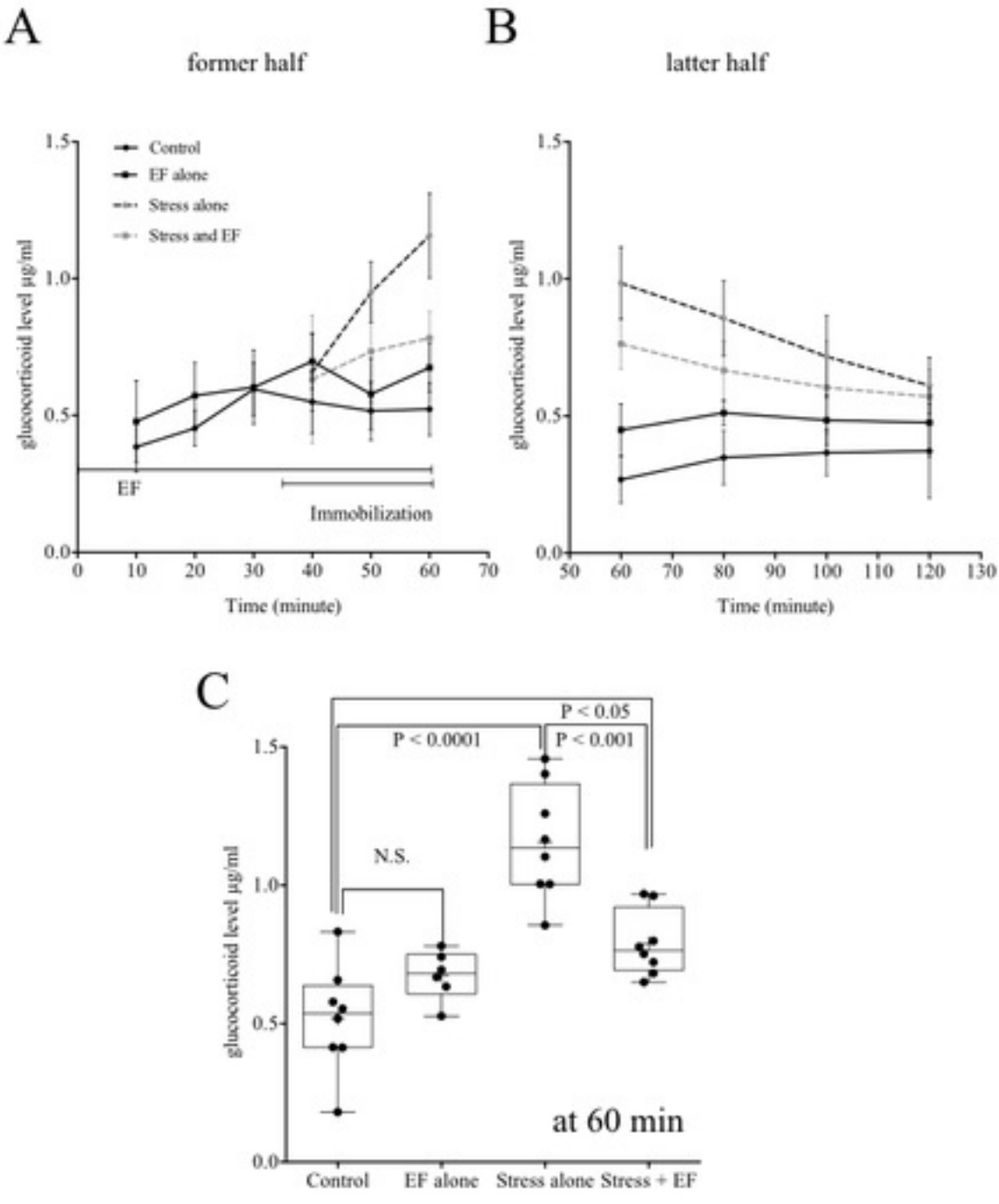
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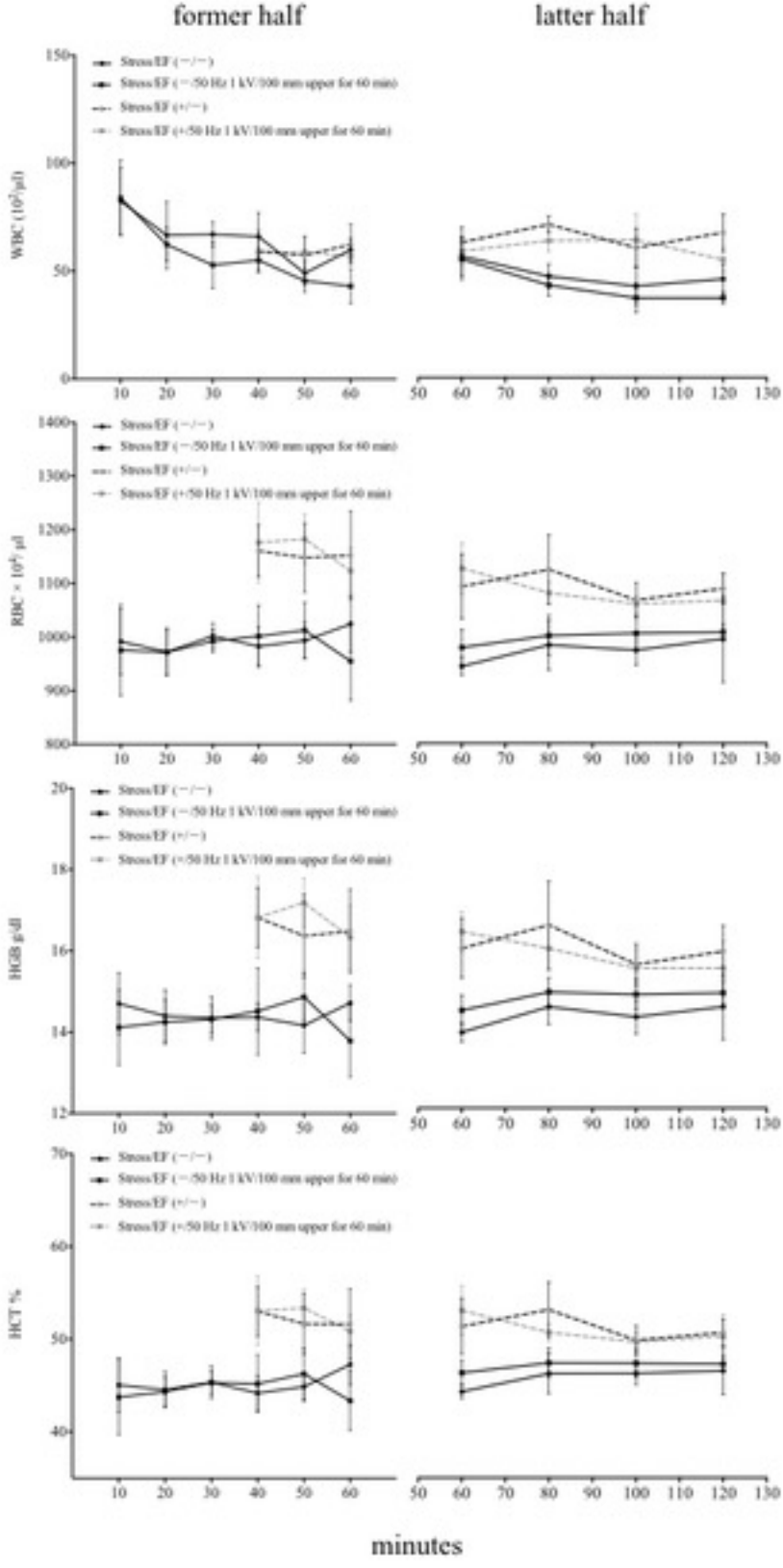
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