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Configuration-dependent variability of the effect of an electric field on the plasma glucocorticoid level in immobilized 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 an immobilization stress-induced increase in the glucocorticoid (GC) level was suppressed in mice exposed to an electric field (EF) of 50 Hz in a kV/m-dependent manner. In the current study, we investigated the reproducibility of the suppressive effect induced by EF exposure by varying the voltage and distance between the electrodes (0.5 kV/50 mm, 1 kV/100 mm, 2 kV/200 mm) and comparing the effects on the plasma GC level. In addition, the effect of the mice being in contact with the lower electrode or not was compared at 1 kV/100 mm. The immobilization-induced GC levels were significantly decreased in mice exposed to an EF at 1 kV/100 mm for 60 min ($P < 0.01$), but not in mice exposed to 0.5 kV/50 mm or 2 kV/200 mm. Furthermore, the suppressive effect of the EF of 1 kV/100 mm was canceled when a polypropylene sheet (0.1 mm thick) was placed between the animal and the lower electrode. Our findings corroborated that an EF of 10 kV/m inhibits stress-induced changes in the endocrine system in mice and demonstrated that this effect depends on the configuration of the EF exposure system, even when the EF strength remains the same.

Keywords: stress; endocrine response; 50 Hz; electrical stimulation; polypropylene

INTRODUCTION

During the last decades, the distribution and use of electricity has increased rapidly, not only in developed but also in developing countries. 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 the 50- and 60-Hz power-line frequencies, even though those are relatively weak [Akpinar D et al., 2012; Gok DK et al., 2016; Hjeresen et al., 1980; Jaffe et al., 1980, 1981; Kantar Gok et al., 2014; Marino et al., 1980; Tiwari et al., 2015; WHO, 2008; Zhang Y et al., 2016]. Induced electric current in the body and the perception of EFs at 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]. Additionally, mechanical vibration of the skin surface at 5–50 Hz, which is similar to the perception of EF exposure, activates neurons in the primary somatosensory cortex that correspond to the site of stimulation [Romo et al., 1998]. Various 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 devices to apply electrical energy to the skin surface, resulting in induced electric currents that mediate desired therapeutic effects [FDA, 2006]. In 1972, the Ministry of Health and Welfare in Japan approved the manufacture of a physical therapy apparatus that uses a 50- or 60-Hz EF to alleviate pain related to shoulder stiffness, insomnia, chronic constipation, and headaches [Hara, 1961]. 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] in addition to cell signaling pathways [Harakawa et al., 2004a]. In addition, *in vitro* studies have suggested that an exogenous EF can induce changes in intracellular calcium ion concentrations and protein synthesis [McLeod et al., 1987; Cho et al., 1999].

In order to understand the biological effects of power line-frequency EFs, it is necessary to first establish experimental methods to assess the quantitative and qualitative effects of exogenous EFs. We previously reported that EF exposure reduced 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, the serum corticosterone concentration was higher than that in 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 interpreted to indicate stress. Thus, the authors concluded that the EF was a stressor.

However, another former study reported reduced blood GC in rats exposed to a 60-Hz, 15-kV/m EF when compared to control rats [Marino et al., 1977]. Furthermore, upon exposure to up to 60 Hz, 50 kV/m induced a reduction in plasma GC concentrations, but only at the beginning of the exposure period [Hackman and Graves, 1981]. Another study revealed no increase in the GC levels after 30–120 days of EF exposure in adult male rats [Free et al., 1981]. GCs are involved in mediating the stress response and are released from the adrenal gland upon ACTH stimulation.

We previously reported that an immobilization-induced increase in the plasma GC level was reduced in BALB/c male mice exposed to an EF of 50 Hz, 10 kV/m [Hori et al., 2015]. In this study, we further investigated the relevance of the configuration of the EF exposure system to the effect of EF exposure on the endocrine stress response.

MATERIALS AND METHODS

Animals

Eight-week-old male BALB/c mice were purchased from Charles River (Tokyo, Japan) and maintained under specific pathogen-free conditions at 24 ± 1 °C, with 50 ± 10% humidity and 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 and Stress Application

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, 2008; Hori et al., 2005, 2015]. The exposure system comprised a cylindrical acrylic cage (diameter, 200 mm; height, 50, 100, or 200 mm) and two stainless steel electrodes (1,000 × 600 mm) that were placed over and under the cylindrical cage and 6 polypropylene pillar spacers (height, 50, 100, or 200 mm) (Fig. 1A–C). The cylindrical cage had slits (100 × 5 mm) all around at 5-mm intervals (Fig. 1B) to prevent smudges (from feces or saliva) from disturbing the formation of a stable EF. To the same end, the

center of the lower electrode was covered with a parallel cross-pattern mesh (280 × 280 mm).

To generate the EF in the cage, 50 Hz, 0.5, 1, or 2 kV was applied to the upper electrode, whereas the lower electrode was grounded. A separate cage and tube were used for each animal; the cages and tubes were reused after they were washed with a neutral detergent and completely dried. To measure the field intensity and to verify the system's operation, an electro-optic voltage meter attached to a digital multimeter (Fluke 87; Fluke, Everett, WA) via a double-core $Bi_{12}SiO_{20}$ fiber (FOVM 03; Sumitomo Electric, Osaka, Japan) was used. The EF intensity was measured at 273 arbitrary points (21 × 13) on each cage floor. 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 between 45/ and 55%. The mice in the EF+ groups were exposed to an EF of 10 kV/m for 60 min. The temperature within the cylindrical cage was 25 ± 3 °C during the period of EF or sham exposure. Stress was applied by immobilizing each mouse separately within a 50-ml centrifuge tube that was placed on the lower electrode (Fig. 1D) [Hori et al., 2015]. Immobilization stress was applied during the second half (30 min) of the 60-min EF test period. All mice spent 60 min in the EF exposure cage.

Analysis of the Effect of Varying Voltage and Distance between the Electrodes

To examine the effect of exposure to the EF alone (i.e., without immobilization stress) on the plasma GC level, ten-week-old mice were divided into two groups (n = 6 per group): A control group Stress/EF (−/−) and an EF-alone group Stress/EF (−/1 kV/100 mm for 60 min).

To examine the effect of the distance between the electrodes on the effect of the EF on the stress-induced plasma GC level, ten-week-old mice (25.8–28.1 g of body weight) were randomized into five treatment groups (n = 8 per group): Stress/EF (–/–), Stress/EF (+/–), Stress/EF (+/0.5 kV/50 mm), Stress/EF (+/1 kV/100 mm), and Stress/EF (+/2 kV/200 mm) (Fig. 2).

Effect of Separation of the Mouse from the Lower Electrode by a Thin Polypropylene Sheet

To assess the influence of direct contact of the mouse with the electrode on the effect of the EF on the stress-induced plasma GC level, ten-week-old mice (25.9–27.9 g of body weight) were randomized into six treatment groups (n = 6 per group): Stress/EF (–/–), Stress/EF (+/–), Stress/EF (+/1 kV/100 mm for 60 min), Stress/EF (+/1 kV/100 mm for 60 min, no direct contact between mouse and lower electrode) (Fig. 3). In the latter treatment, the lower electrode was covered with a polypropylene sheet of 0.1, 1, or 5 mm thickness.

Derivatization and Fluorimetric Assay of Plasma Glucocorticoid Levels

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

To derivatize the GC, 200 µl of plasma was mixed with 900 µl of isooctane (2,2,4-trimethylpentane; Wako, Osaka, Japan). The mixture was vortexed and centrifuged at 380 × *g* at room temperature for 5 min. The supernatant was discarded, and 900 µl of chloroform (Wako) was added to the sample. The mixture was vortexed and centrifuged at 380 × *g* at room temperature for 5 min. The supernatant and the white membranous layer 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 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 using Tukey's multiple comparison test. Interactions between the plasma GC level and the thickness of polyethylene sheet 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

There were no significant differences in plasma GC between mice that were exposed to the EF but not immobilized (50 Hz, 1 kV/100 mm for 60 min) and the mice in the control group (data not shown). GC levels were significantly higher in the group including immobilized mouse than in the Stress/EF (–/–) group ($P < 0.0001$), while they were lower in the Stress/EF (+/1 kV/100 mm) than in the Stress/EF (+/–) group ($P < 0.0005$). GC levels in the Stress/EF (+/0.5 kV/50 mm) and (+/2 kV/200 mm) groups did not show significant differences when compared to the level in the Stress/EF (+/–) group. GC levels in the Stress/EF (+/0.5 kV/50 mm) group were not significantly different from those in the Stress/EF (+/1 kV/100 mm), and the levels in the Stress/EF (+/2 kV/200 mm) group were significantly higher than those in the Stress/EF (+/1 kV/100 mm) ($P < 0.001$) (Fig. 4). GC levels were significantly higher in the groups including immobilized mouse than in the Stress/EF (–/–) group ($P < 0.001$).

They were lower in the Stress/EF (+/1 kV/100 mm) than in the Stress/EF (+/–) group ($P < 0.005$). GC in the groups separated from the electrode by a polypropylene sheet, Stress/EF (+/1 kV/100 mm, separated with a 0.1-, 1-, or 5-mm-thick sheet) did not significantly differ from that in the Stress/EF (+/–) group (Fig. 5). GC levels were lower in the Stress/EF (+/1 kV/100 mm) than in the Stress/EF (+/1 kV/100 mm, separated with a 0.1- or 1-mm-thick sheet) groups ($P < 0.005$). There was no significant correlation between sheet thickness and GC level (Supplemental Figure 1).

DISCUSSION

In this study, we examined the reproducibility of the suppressive effect induced by EF exposure. Plasma GC increased approximately 5-fold after immobilization, suggesting that the immobilization procedure activated the endocrine system of the pituitary-adrenocortical axis and/or the sympathetic-adrenomedullary system, indicating stress [Kvetnansky et al., 1979; Sudo and Miki, 1993; Yamada et al., 1996; Arakawa et al., 1997]. Exposure to the EF suppressed the increase in GC in immobilized mice, indicating that the EF has an anti-stress effect and/or a suppressive effect on GC secretion. These findings are consistent with those of our previous studies, which revealed that the elevation in the level 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 an immobilization-induced increase in GC is suppressed by a 50-Hz EF at 10 kV/m in mice [Hori et al., 2015]. In the study by de Bruyn and de Jager [1994], the plasma level of corticosterone (41.9 ± 22.8 ng/ml) in 6-month-old male mice increased approximately 3.26 times in mice exposed to 10 kV/m for 22 h. In our study, the GC level was 0.26 ± 0.11 μg/ml in the control group and 0.32 ± 0.12 μg/ml in the EF alone group (1.23 times higher). Thus, the hormone level under EF treatment seems to be higher than that under sham treatment; however, our data did not reveal a significant difference. Therefore, the change induced by the EF is very small in comparison to that induced by immobilization. Plasma levels of GC in the mice were 0.19 μg/ml at 9:00, 0.28 μg/ml at 11:00, 0.39 μg/ml at 12:00, and 0.45 μg/ml at 15:00, suggesting circadian changes in plasma GC. However, the range of change is relatively small compared to that between the stress and stress/EF groups.

In the current study, we applied a standard EF of 50 Hz 1 kV/100 mm (10 kV/m) for 60 min because our previous study showed that EFs at > 5 kV/100 mm (50 kV/m) generated side effects such as vibration and/or noise, and that data on GC levels recorded at ≥ 50 kV/m might be affected by both EF exposure and vibration [Hori et al., 2015]. Our results corroborated that exposure to a 50-Hz, 1-kV/100 mm (10-kV/m) EF for 60 min suppresses the immobilization-induced GC increase in BALB/c mice. As there was no correlation between body weight and GC

level in the Stress/EF (–/–), Stress/EF (+/–), and Stress/EF (+/1 kV/100 mm) groups, body mass did not influence the effect of EF in this study. Further, the data indicated that the conditions of EF exposure should be optimal in order to observe its effect on stress-induced hormonal changes in immobilized BALB/c mice. Therefore, the experimental system described in this report may be applied in other studies on the effect of an EF on mice.

Although exposure to an EF of 1 kV/100 mm (10 kV/m) showed the GC-suppressive effect with high reproducibility, exposure to 2 kV/200 mm (10 kV/m) and 0.5 kV/50 mm (10 kV/m) did not have this effect. This means that the configuration of the EF exposure system possibly influences the effect. In addition, because the effect of the EF depended on the distance between the animal and the upper electrode, the animal size might have an influence. Therefore, fine-tuning of the voltage or distance between the electrodes would be needed to create the same EF effect on animals of different size. Further, because the EF contribution is expected to change depending on the position of the mouse (standing or sitting), time-dependent changes in EF contribution should be monitored to elucidate our findings.

A 0.1-mm thick polypropylene sheet placed between the lower electrode and the animal completely annihilated the suppressive effect of the EF on the stress-induced GC increase. It is generally accepted that 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]. However, we found that even a slight vibration of the hairs during exposure annihilated the effect of the EF during immobilization (unpublished data). It is conceivable that for the anti-stress effect of the EF to be effective, the mouse has to be in direct contact with the electrode. In general, it has been considered that a current induced by an external field would trigger a biological effect. Computed [Gandhi and Chen, 1992] and measured [Deno, 1977] current distributions have been reported for an ungrounded and grounded human of 1.77 m in height, standing in a vertical homogeneous electric field of 50 Hz at 10 kV/m. The data showed that the mean induced current in the grounded body was than that in the ungrounded body. This seems to be consistent with our finding that the anti-stress effect was suppressed in ungrounded mice, even though under the same strength of EF. On the other hand, a 5-mm gap between the animal and the lower electrode tended to restore the EF effect, indicating that some kind of electrical phenomenon including a weak electrical shock might have occurred between the mouse and the lower electrode in case of the 0.1-mm gap, but not in case of the 5-mm gap. We suppose that such an electrical event might occur between the tip of a nail, hair, or the tongue and the electrode. Future studies are required to confirm this hypothesis.

In conclusion, although there are some limitations to our study, e.g., only one species was tested and the effect of animal size was not investigated, our findings corroborated that exposure to a low-frequency EF suppresses immobilization-induced changes in the endocrine system and suggest that the effect of an EF in mice is greatly influenced by the EF exposure conditions including the distance between the animal and the electrodes, even when the EF strength remains the same.

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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 effect of varying the voltage and distance between the upper and lower electrodes. The mice were divided into five treatment groups indicated by separate boxes. Stress/EF (+/+) mice were exposed to the EF for 60 min and were immobilized during the second half (30 min) of the EF exposure period. In the control group, mice were handled in an identical manner except that the EF was 0 V/m, and they were not immobilized.

Fig. 3. Experimental design used to investigate the influence of separation of the mouse from the lower electrode during EF exposure.

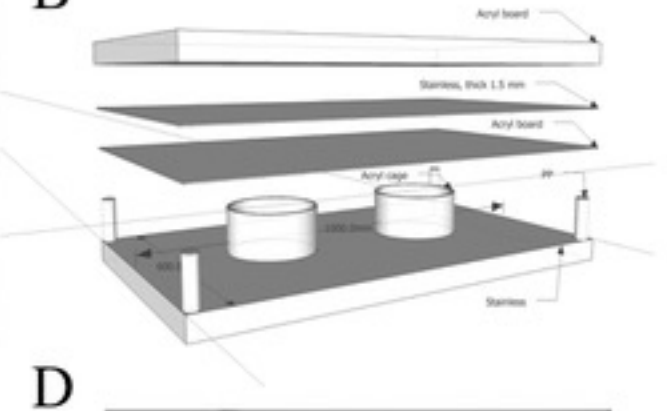
Fig. 4. Effect of immobilization on the plasma GC level and effect of the EF on the immobilization-induced increase in plasma GC.

Fig. 5. Effect of separation of the animal from the lower electrode during exposure to the EF on the immobilization-induced increase in plasma GC.

Supplemental Fig. 1. Pearson correlation analysis between thickness of the polypropylene sheet and the GC level. No significant correlation was observed.

A

B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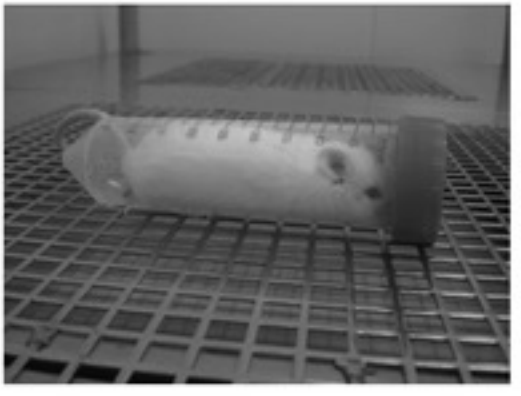
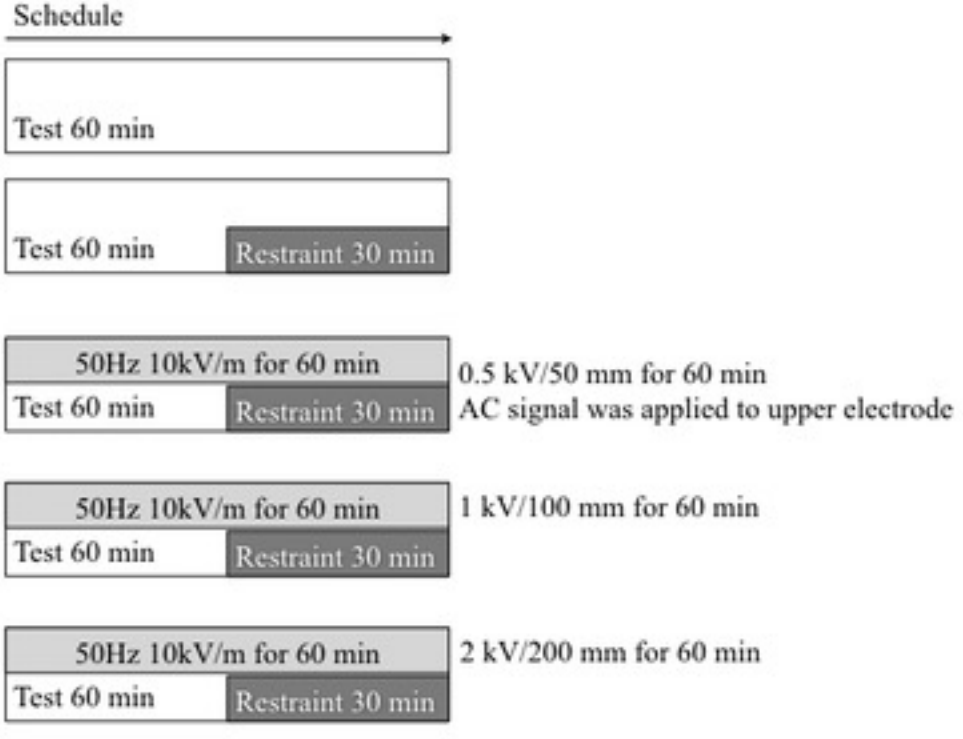
Stainless, thick 1.5 mm
Acryl board
Acryl cage
Stainless

C

D

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Test 60 min
Restraint 30 min
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Test 60 min
Restraint 30 min
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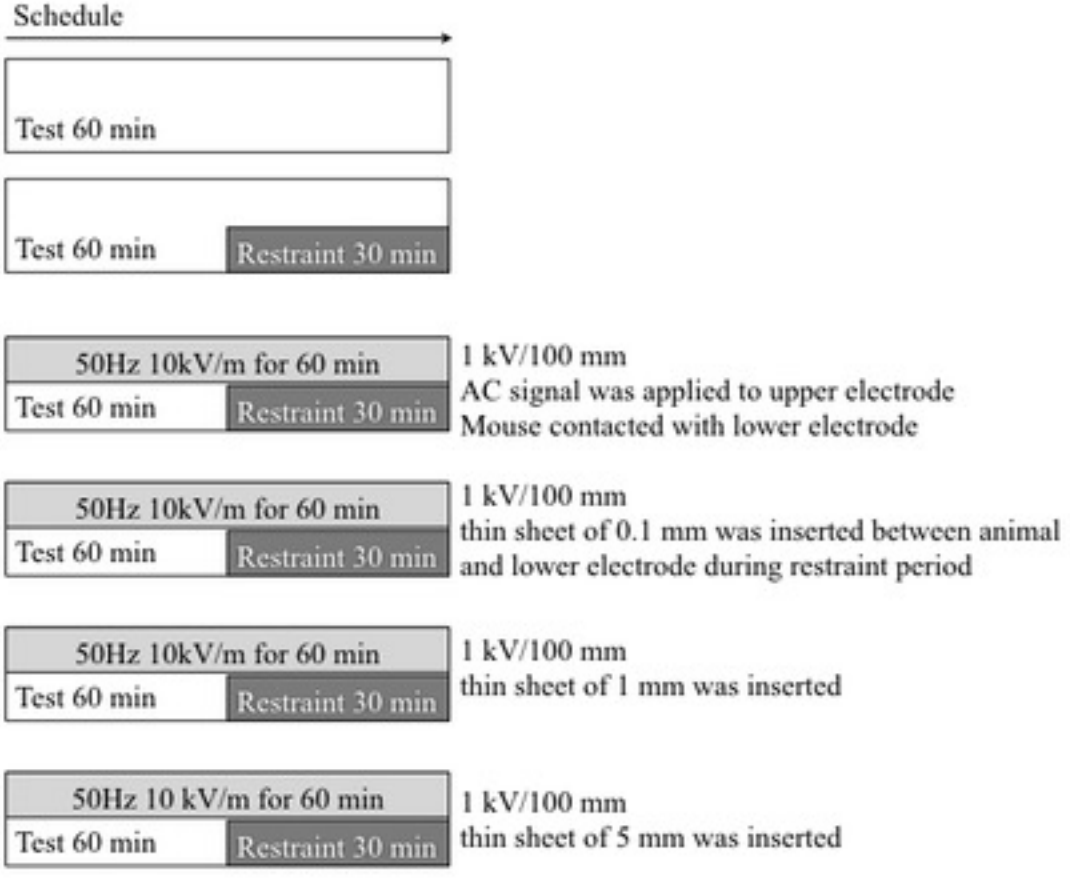
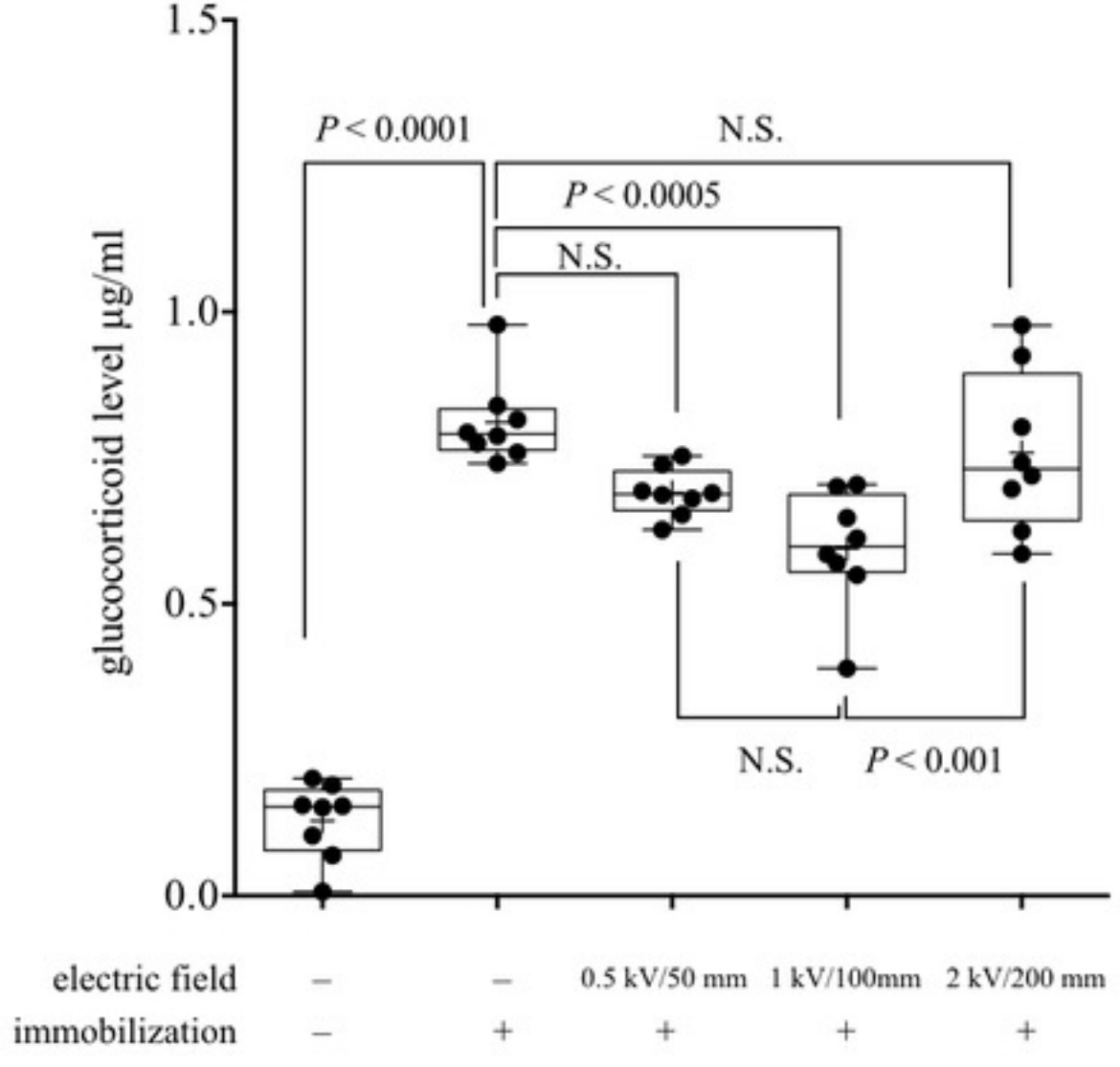
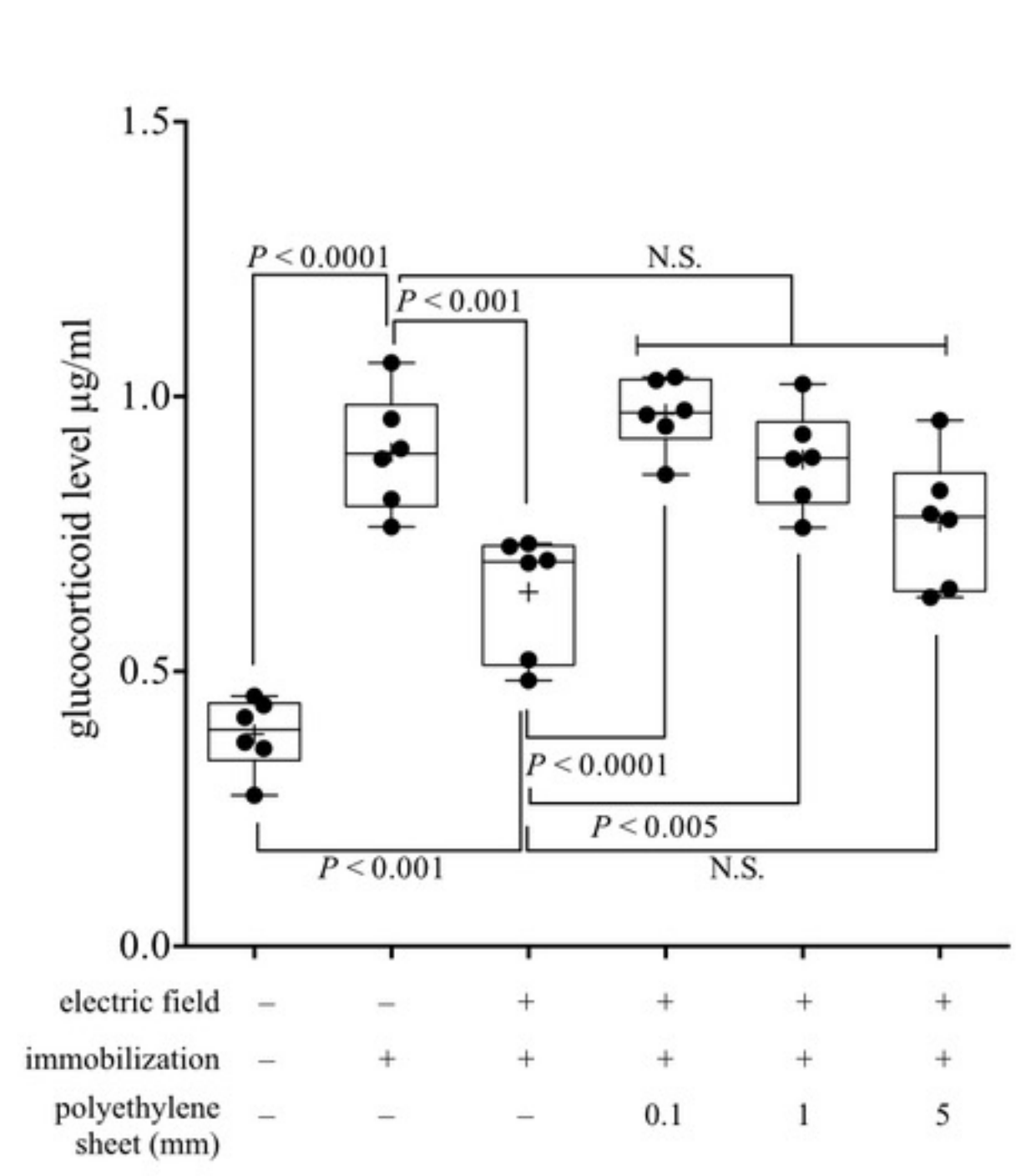
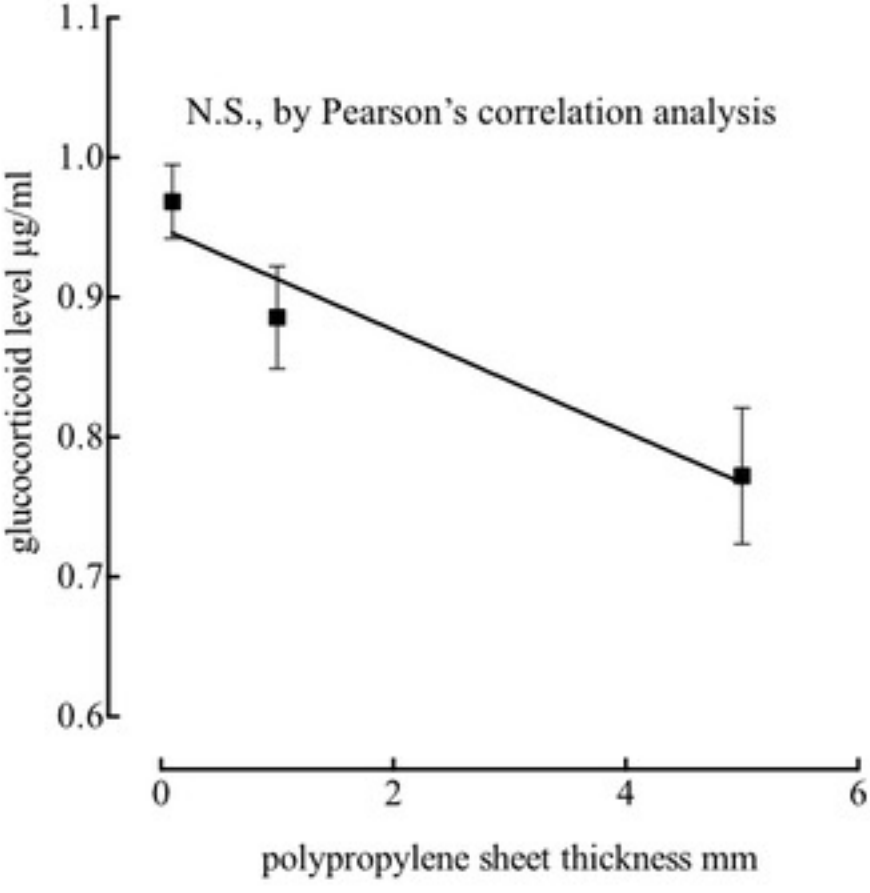
AC signal was applied to upper electrode
50Hz 10kV/m for 60 min
Test 60 min
Restraint 30 min
1 kV/100 mm for 60 min
50Hz 10kV/m for 60 min
Test 60 min
Restraint 30 min
2 kV/200 mm for 60 min


Schedule
Test 60 min
Test 60 min
Restraint 30 min
50Hz 10kV/m for 60 min
Test 60 min
Restraint 30 min
1 kV/100 mm
AC signal was applied to upper electrode
Mouse contacted with lower electrode
50Hz 10kV/m for 60 min
Test 60 min
Restraint 30 min
1 kV/100 mm
thin sheet of 0.1 mm was inserted between animal and lower electrode during restraint period
50Hz 10kV/m for 60 min
Test 60 min
Restraint 30 min
1 kV/100 mm
thin sheet of 1 mm was inserted
50Hz 10 kV/m for 60 min
Test 60 min
Restraint 30 min
1 kV/100 mm
thin sheet of 5 mm was inserted


1.5
1.0
0.5
0.0
glucocorticoid level μg/ml
P < 0.0001
N.S.
P < 0.0005
N.S.
N.S.
P < 0.001
electric field
immobilization
–
–
+
0.5 kV/50 mm
+
1 kV/100mm
+
2 kV/200 mm
+


1.5
1.0
0.5
0.0
glucocorticoid level μg/ml
P < 0.0001
N.S.
P < 0.001
P < 0.0001
P < 0.005
P < 0.001
N.S.
electric field – – + + + +
immobilization – + + + + +
polyethylene sheet (mm) – – – 0.1 1 5


N.S., by Pearson's correlation analysis
glucocorticoid level μg/ml
1.1
1.0
0.9
0.8
0.7
0.6
0
2
4
6
polypropylene sheet thickness mm