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Effect of Maternal Electroacupuncture on Perinatal Nicotine Exposure-Induced Lung Phenotype in Offspring

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Abstract

Introduction—Pregnant women exposed to tobacco smoke predispose the offspring to many adverse consequences including an altered lung development and function. There is no effective therapeutic intervention to block the effects of smoke exposure on the developing lung. Clinical and animal studies demonstrate that acupuncture can modulate a variety of pathophysiological processes, including those involving the respiratory system; however, whether acupuncture affects the lung damage caused by perinatal smoke exposure is not known.

Methods—To determine the effect of acupuncture on perinatal nicotine exposure on the developing lung, pregnant rat dams were administered (1) Saline; (2) Nicotine; or (3) Nicotine + electroacupuncture (EA). Nicotine was administered (1 mg/kg subcutaneously) once a day and EA was applied to both “Zusanli” (ST 36) points. Both interventions were administered from gestational day 6 to postnatal day 21 (PND21), following which pups were sacrificed. Lungs, blood and brain were collected to examine the markers of lung injury, repair and hypothalamic pituitary adrenal (HPA) axis.

Results—Concomitant EA application blocked nicotine-induced changes in lung morphology, lung Peroxisome Proliferator-Activated Receptor γ and Wingless-int signaling, two key lung developmental signaling pathways, hypothalamic pituitary adrenal axis (hypothalamic corticotrophic releasing hormone and lung glucocorticoid receptor levels), and plasma β -endorphin levels.

Conclusions—Electroacupuncture blocks the nicotine-induced changes in lung developmental signaling pathways and the resultant myogenic lung phenotype, known to be present in the affected offspring. We conclude that EA is a promising novel intervention against the smoke exposed lung damage to the developing lung.

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Conflict of Interests

The authors declare no financial conflict of interest regarding the publication of this work.

Keywords

Nicotine; Smoking; Electroacupuncture; Lung development; PPAR γ ; Wnt Signaling

Introduction

Pregnant women who smoke or are exposed to environmental tobacco smoke predispose the offspring to many adverse consequences. Some of these include spontaneous abortion [1], preterm birth [2], stillbirth [3], fetal growth restriction [4], low birth weight [5], lung hypoplasia and asthma [6], sudden infant death syndrome [7], etc. Previous studies have suggested that nicotine, one of the more than 4000 ingredients of tobacco smoke, is a key factor in causing some of the adverse outcomes noted above. It rapidly crosses the placental barrier with minimal biotransformation, accumulating in the amniotic fluid and various organs of the fetus including the developing lung and brain [8]. Although a small to moderate nicotine exposure during pregnancy does not cause death of the fetus, it potentially affects the development of multiple organ systems in the fetus, especially the respiratory system - this effect appears to be permanent, sustainable to adolescence and adulthood [9–12], and in fact passed not only to the exposed offspring, but also transgenerationally [13–15].

The detrimental effects of nicotine on the alveolar formation and airway development, at least in part, are mediated by the up-regulation of the myogenic mesenchymal Wingless-int (Wnt) signaling and the down-regulation of the homeostatic Peroxisome Proliferator-Activated Receptor γ (PPAR γ) signaling [16]. This Wnt and PPAR γ signaling imbalance is initiated following nicotine binding to its receptor nicotinic acetylcholine $\alpha 7$ [17], resulting in an altered lung developmental programming. Although using a targeted postnatal intervention, we have recently demonstrated reversibility of the perinatal nicotine-induced lung phenotype [18]; however, short of the cessation of smoking during pregnancy (<http://www.cdc.gov/tobacco>), which is not always achievable, a more practical strategy would be to safely and proactively block perinatal nicotine-induced lung damage before it occurs. Clinical and animal studies have demonstrated that acupuncture modulates a variety of the body's patho-physiological responses, including hypothalamic-pituitary-adrenal (HPA) axis, immune function, energy metabolism, and microcirculation, potentially affecting the development of many organ systems including the respiratory tract [19–23]; however, whether acupuncture has a protective effect on the lung damage caused by perinatal nicotine exposure is not known. We hypothesized that by modulating key regulators of lung development, acupuncture blocks the development of the perinatal nicotine-induced lung phenotype. Therefore, we aimed to determine the effect of perinatal nicotine exposure on the developing lung in the presence or absence of acupuncture.

For acupuncture application, we choose “Zusanli” (ST 36), which is one of the 365 classical acupuncture points. It's a He-sea point of the “Stomach Meridian”, located on the leg (Fig. 1) and is one of the most frequently used acupuncture point. It's easily reproducible and, in addition to its effects on the digestive system, it is claimed to be effective in multiple other system diseases, including pulmonary conditions such as wheezing, asthma, and dyspnea

[24–26]. Therefore, we opted to use “Zusanli” (ST 36) to counteract the effects of perinatal nicotine exposure on the developing lung.

Materials and Methods

Experimental Animals

This experimental protocol was approved by the Beijing University of Chinese Medicine Ethics Committee on April 13, 2012 (approval number: 2012-040). All animal experiments were performed in compliance with the United States National Institutes of Health Advocacy, using the guidelines of Reduction, Replacement, and Refinement for animal experiments (“3R” principle). Twelve female (9-week old, 220 ± 20 g) and nine male (9-week old, 220 ± 20 g) clean grade Sprague-Dawley rats of sexual maturity but without history of previous mating were acquired from Beijing Weitong Lihua Experimental Animal Technical Co., Ltd. (Beijing, China). Rearing conditions included: indoor temperature $23 \pm 1^\circ\text{C}$, humidity $45 \pm 5\%$, regular disinfection of the feeding cages and water bottles, provision of general rat chow and free drinking water, and rat weighing once every 7 days. The rats were maintained on a 12h light-dark cycle.

Experimental Equipment and Reagents

Nicotine was purchased from Sigma-Aldrich, St. Louis, MO (Catalog #: N590200-25mg); corticotropin-releasing hormone (CRH, Catalog #: CSB-E08038r), PPAR γ (Catalog #: CSB-E08624r), SP-A (Catalog #: CSB-E08684r), and SP-B (Catalog #: CSB-E12630r). Enzyme linked immunosorbent assay (ELISA) kits were purchased from Cusabio Biotech Co., Ltd. Acupuncture equipment used included 0.25 mm * 26 mm HuaTuo acupuncture needles, obtained from Suzhou Medical Appliance Factory Co., Ltd and LH202H Han’s Acupoint Neuro Stimulating Instrument (Beijing Huawei Industry Development Co., Ltd). Image-pro Plus 5.0 Image analysis system (Olympus Co., Ltd, Japan) was used for analyzing morphometric data.

Experimental Protocol

The experiments started one week after acclimatization period of animals. Two female rats randomly cohabitated with one male rat in the evening, and on the second morning, vagina was washed with saline, smeared, and examined for sperms under a microscope (magnification: 40 $\times$). Detection of sperms suggested mating and possible fertilization. Nicotine injections were started on embryonic day (E) 6, continued during pregnancy and following delivery up to postnatal day (PND) 21, as described by us previously [13, 14, 22, 27]. During the experimental period, animals were pair-fed according to the intake of nicotine exposed animals; food and water intake, body weight, and the general well-being-related indicators were closely monitored.

The dams were grouped as follows: (1) Saline group: saline was injected subcutaneously (s.c.) in 100 μ l volume once a day; (2) Nicotine group: nicotine was injected s.c., 1 mg/kg body weight/day in 100 μ l volumes; (3) Nicotine+ Electro-acupuncture (EA) group: nicotine was injected s.c., 1 mg/kg body weight/day in 100 μ l volumes and EA was applied to both “Zusanli” (ST 36) points. A subgroup of animals was treated with saline+EA at either acu-

or non-acupoint locations without nicotine administration, i.e., saline was injected s.c., 100 μ l once a day and EA was applied to either both “Zusanli” (ST 36) points or at nearby non-acupoint locations in the leg. All treatments started on E6 were carried up to PND21.

Electro-acupuncture Treatment

On the basis of the morphological, anatomical, and physiological characteristics of the rat model and referring to Lu-fen Zhang’s “Experimental Acupuncture Science” [28], “Zusanli” (ST 36) was selected for EA (Fig. 1). Rat dams, held in a locally made restraining bag, with head and legs outside of the bag, at opposite ends, were taped in prone position on a tabletop. When the dam was quiet, acupuncture needles, 0.25 mm * 26 mm in size, were perpendicularly inserted into “Zusanli” (ST 36) points at a depth of 4–5 mm and then 1 mA electric current with 2/15 Hz frequency was applied for 20 min. The negative pole was connected to the right, and the positive pole was connected to the left “Zusanli” point. As stated above, EA was applied once every day from E6 until the pups were delivered spontaneously at term and then up to PND 21, for a total of 36–37 sessions.

Sample Collection and Processing

Sample collection—At the end of the experimental period, dams and pups were killed; pups’ lungs, brain, and blood were collected for analysis. Serum was separated by centrifugation at 2,000 $\times g$ at 4°C for 10 min and samples frozen at –80°C until processing. Left lung and hypothalamus were flash-frozen in liquid nitrogen for later processing. For bronchoalveolar lavage (BAL) collection, a total of three separate 1 ml aliquots of sterile phosphate-buffered saline (PBS) containing 5 mM ethylenediaminetetraacetic acid, 5 mM dithiothreitol, and 5 mM phenylmethylsulfonyl fluoride were instilled and then collected through a tracheal catheter. BAL was immediately centrifuged at 1,500 $\times g$ and supernatant frozen in liquid nitrogen for subsequent ELISA assays.

Lung tissue morphology—For lung perfusion, pups were dissected open, and trachea cannulated and perfused with 4% paraformaldehyde (PFA) in PBS, at a pressure of 20 cm of water. Following ligation, lungs along with the heart were removed and submerged in 4% PFA for 4–5 hrs, followed by immersion in 30% sucrose in PBS. Following submersion in sucrose, the tissue was paraffin-embedded. Lung morphometry was performed on 5- μ m thickness hematoxylin and eosin-stained tissue slices, using the previously described methods [29, 30].

Western analysis—Western analysis on protein lysates from isolated lungs was performed according to previously described methods [13, 14, 27, 29]. The protein concentration of the supernatant was measured by the Bradford method, using bovine serum albumin as the standard. Aliquots of the supernatant, each containing 50 μ g of protein, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrically transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by incubation with Tris-Buffered Saline (TBS) containing 5% nonfat dry powdered milk (w/v) for 1h at room temperature. After a brief rinse with TBS containing 0.1% tween 20 (TBST), the protein blots were incubated in primary antibody (PPAR γ , 1:500, Santa Cruz, Cat #: sc-7196; ADRP, 1:250, Santa Cruz #: sc-32888; β -Catenin 1:300, Santa Cruz, #: sc-7963;

LEF-1, 1:500 Santa Cruz #: sc-28687; α -SMA 1:5,000, Sigma, #: A2547; Calponin, 1:3,000, Sigma, #: C2687; Glucocorticoid Receptor (GR), 1:200, Santa Cruz, #: sc-8992; or GAPDH, 1:4,000, Millipore, Cat. #: MAB374), overnight at 4°C followed by incubation with an appropriate secondary antibody for 1h at room temperature. After three more washes in TBST, the blots were exposed to X-ray film using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and developed. The relative densities of the protein bands were determined with UN-SCAN-IT software (Silk Scientific Inc, Orem, Utah), and normalized to that of GAPDH.

Immunofluorescence staining

For tissue immunofluorescence staining for the relevant proteins, rat lungs were inflated *in situ* with 4% PFA in PBS at a standard inflation pressure of 20 cm H₂O for 4h at 4°C. The lungs were subsequently transferred to PBS containing 30% sucrose (wt/vol) until equilibrated in the cold (4°C). After fixation, 5- μ m paraffin sections were treated three times with Histo-ClearTM (National Diagnostics, Atlanta, GA) for 5 min, and then rehydrated by a sequential ethanol wash. Sections were then washed twice for 10 min with PBS, and blocked for 1h in PBS-5% normal goat serum-0.2% Triton X-100. Sections were incubated with primary antibodies for 1h at room temperature, and then with the appropriate secondary antibody for 30 min, also at room temperature. Antibodies used included PPAR γ , 1:150, Santa Cruz, #: sc-7196, secondary antibody, Alexa Fluor goat anti rabbit 568, 1:100 (red); β -catenin, 1:100, Santa Cruz, #: sc-7963, secondary antibody, Alexa Fluor anti mouse 488, 1:100 (green); α -SMA, 1:500, Sigma-Aldrich, #: A2547, secondary antibody, Alexa Fluor goat anti mouse 488, 1:200 (green); calponin 1:250 Sigma-Aldrich, #:C2687, secondary antibody, Alexa Fluor goat anti mouse 568, 1:150 (red); GR, 1:100, Santa Cruz, #: sc-8992, secondary antibody, Alexa Fluor anti rabbit 568, 1:100 (red). Then, sections were washed with PBS and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) for visualization under a fluorescence microscope.

ELISA Assay for lung PPAR γ and β -catenin, hypothalamic CRH, and BAL SP-A and SP-B levels—All ELISA assays were performed following manufacturer's instructions. For lung PPAR γ and β -catenin levels, flash-frozen left lung tissue was homogenized and ELISA performed on the supernatant. For hypothalamic CRH levels, hypothalamic tissue was homogenized and ELISA performed on the supernatant. Similarly, BAL SP-A and SP-B levels were determined using ELISA on the BAL supernatant.

Statistics

All data are presented by mean $\pm$ standard error (SE). The difference between each group was analyzed by "One-Way ANOVA", followed by post-hoc analysis using Bonferroni correction. A *p*-value of < 0.05 was considered significant.

Results

Initially, using lung morphometry, lung PPAR γ and β -catenin, and BAL SP-A and SP-B protein levels, as markers of lung injury and repair, two subgroups of animals (n=3–4 for each) were used to assess the effect of sham acupuncture (acupuncture using non-acupoints)

and EA alone (without nicotine) on lung development,. There were no differences in any of the aforementioned parameters between these two groups vs. the saline control group (data not shown); hence all subsequent data are presented only for the control (Saline), Nicotine, and Nicotine + EA groups.

Effect of Electroacupuncture on Perinatal Nicotine-Induced Changes in Lung Morphometry

As shown in Fig. 2, compared to the control group, in line with our previous data [13, 14, 18, 27], animals from the perinatal nicotine exposure group demonstrated larger alveoli (increased mean linear intercept), decreased alveolarization (lower alveolar count), and increased septal thickness. The morphology in nicotine + EA group showed normalization of the nicotine-induced changes, with the mean linear intercept, radial alveolar count, and septal thickness being not significantly different from the control (Saline) group.

Effect of Electroacupuncture on Perinatal Nicotine-Induced Changes in Lung PPAR γ and Wnt signaling

Since the perinatal nicotine-induced lung phenotype has been shown to at least partially result from imbalanced PPAR γ and Wnt signaling [13, 14, 16, 18, 27], we next probed for key markers of these signaling pathways. Consistent with the previously published data, perinatal nicotine exposure resulted in decreased PPAR γ signaling, as indicated by decreased PPAR γ (Western analysis and immunostaining) and its downstream target adipocyte differentiation-related protein (ADRP) (Western analysis) levels, and blockage of these changes by the concomitant EA treatment (Fig. 3A). Furthermore, perinatal nicotine exposure-induced up-regulation of β -catenin (Western analysis, immunostaining) and LEF-1 (Western analysis), two key mediators of Wnt signaling, was also blocked in the EA-treated group (Fig. 3B). The blockage of nicotine-induced changes in whole lung PPAR γ and β -catenin levels was also supported by ELISA (Fig. 4). In fact, as indicated by ELISA, EA not only resulted in blockage of the nicotine-induced decrease in whole lung PPAR γ levels, but resulted in significantly higher levels than the nicotine exposed group. The increase in myogenic proteins α -SMA and calponin (Western analysis), which is a known consequence of activated Wnt signaling were also blocked by concomitant EA administration (Fig. 5A and 5B).

Effect of Electroacupuncture on Perinatal Nicotine-Induced Changes in BAL SP-A and SP-B Levels

We next examined SP-A and SP-B levels (ELISA), two well-established lung functional determinants. Nicotine treatment resulted in a significant increase in BAL SP-A levels, with blockage of this effect in the concomitantly EA-treated group (Fig. 6A). In contrast, the BAL SP-B content in the nicotine exposed group decreased significantly, which was also normalized in the concomitantly EA-treated group (Fig. 6B).

Effect of Electroacupuncture on Perinatal Nicotine-Induced Changes in Key Markers of HPA axis

Since fetal lung development is driven by HPA axis and acupuncture is known to modulate HPA axis [31–33], we next determined hypothalamic CRH and its downstream target relevant for lung development, i.e., lung GR levels. Compared to the saline only-administered group, hypothalamic CRH content (ELISA) in the nicotine-treated group decreased significantly; however, concomitant EA treatment blocked this decrease (Fig. 7A). In contrast, lung GR levels increased in the nicotine exposed group, an effect that was also normalized in the EA-treated group (Fig. 7B and 7C).

Effect of Electroacupuncture on Perinatal Nicotine-Induced Changes in Circulating β -endorphin Levels

Lastly, since CRH regulates β -endorphin levels, an important marker of body's stress response, which is a key factor in determining lung development and is known to be affected by acupuncture [34], we next determined the effect of perinatal nicotine exposure and EA on plasma β -endorphin levels. In line with the other data on HPA axis (hypothalamic CRH and lung GR levels), concomitant EA administration blocked perinatal nicotine-induced decrease in plasma β -endorphin levels (Fig. 8).

Discussion

Our data demonstrates that concomitant EA normalizes the perinatal nicotine-induced changes in 1) lung morphometry; 2) key lung developmental signaling pathways, i.e., the down-regulation of PPAR γ and the upregulation of Wnt signaling; 3) HPA axis, i.e., the decrease in hypothalamic CRH and the increase in lung GR levels; and 4) plasma β -endorphin levels, which were reduced in the nicotine-exposed group. These data suggest that EA blocks nicotine-induced changes in key modulators of lung development and the resultant myogenic lung phenotype that is known to be associated with asthma and other forms of CLD in the affected offspring [16]. Furthermore, it's important to note that there was no effect of sham acupuncture on the examined lung morphometry and lung maturation markers (lung PPAR γ and β -catenin, and BAL SP-A and SP-B levels).

Acupuncture affects many pathophysiological processes and has been shown to benefit many diseases [35–39]. In general, acupuncture modulates visceral function by stimulating acupoint receptors, signaling to the spinal cord and brain through afferent nerves, followed by modulation of the homeostatic central and peripheral responses (3). While it may modulate organ function directly via the central nervous system efferent output, it can also elicit these effects via the neuro-endocrine axis. Therefore, by affecting a variety of responses, acupuncture can potentially affect the developing respiratory system. For example, it modulates the HPA axis, endorphin secretion, and circulating prostaglandin E2 levels, all known modulators of lung development; therefore, it's not surprising that in line with our data, acupuncture has also been previously shown to ameliorate other forms of lung injuries [21, 39].

In line with previous studies, we found that perinatal nicotine exposure resulted in decreased alveolarization, larger alveoli and thicker alveolar walls. Concomitant EA application blocked these changes to a large extent. Similarly, nicotine-induced changes in HPA axis markers, developmentally relevant alveolar signaling pathways, and functional lung maturation markers were also blocked by Zusanli EA. Though the underlying mechanisms involved in the protection afforded by EA need to be further explored, the blockage of nicotine-induced changes in key HPA axis modulator CRH and its downstream target, e.g., GR in the developing lung, and plasma β -endorphin levels, suggests a centrally-mediated phenomenon.

Conclusion

In conclusion, concomitant EA “Zusanli” (ST 36) application effectively blocked the perinatal nicotine-induced lung phenotype in the exposed offspring, as determined by the normalization of lung morphometric and molecular changes, as well as the modulators of lung development, e.g., CRH and β -endorphin levels. Furthermore, we speculate that benefits of our approach are probably not restricted only to perinatal nicotine exposure, but are likely to be much wider. We anticipate that this approach might also block the adverse consequences of altered fetal programming secondary to a variety of other stresses, e.g., nutritional stress, i.e., Barker hypothesis [40]. As it's difficult to design interventions that fundamentally revert the effects of developmental programming post-hoc, we argue that active intervention via acupuncture during the antenatal, natal, and/or immediate postnatal periods would be a better and more effective strategy in preventing the altered developmental programming in the first place.

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Fig. 1.
Arrow point to the area of “Zusanli” (ST 36).

Fig. 2. Effect of electro-acupuncture on perinatal nicotine exposure-induced changes in lung morphometry

A: Representative hematoxylin-eosin-stained lung sections from different experimental groups are shown (magnification $\times 20$). Compared with saline only group, nicotine administration increased mean linear intercept (A), decreased alveolar count (B), and increased septal thickness (C); all of these changes were blocked in the nicotine + EA treated group. Values are mean $\pm$ SE; $n=6$ for each group. * $p < 0.05$ vs. saline; # $p < 0.05$ vs. nicotine.

Fig. 3. Effect of electro-acupuncture on perinatal nicotine-induced changes in lung PPAR γ and Wnt signaling

Perinatal nicotine exposure resulted in decreased PPAR γ signaling, as indicated by decreased PPAR γ (Western, immunostaining) and its downstream target adipocyte-differentiation-related protein (ADRP) (Western analysis) levels. Concomitant EA treatment blocked these changes (3A). Similarly, perinatal nicotine exposure induced up-regulation of β -catenin (Western analysis and immunostaining) and LEF-1 (Western analysis) was blocked by concomitant EA administration (3B). Representative lung sections for PPAR γ (3A) and β -catenin (3B) immunostaining are shown. Values are mean $\pm$ SE; $n=6$ for each group. * p < 0.05 vs. saline; # p < 0.05 vs. nicotine.

Fig. 4. Effect of electro-acupuncture on perinatal nicotine-induced changes in lung PPAR γ and β -catenin levels

As determined by ELISA, compared with the control group, perinatal nicotine exposure resulted in a decrease in PPAR γ (A), but an increase in β -catenin levels (B); concomitant EA treatment blocked both of these effects. Values are mean $\pm$ SE; $n=6$ for each group. * $p < 0.05$ vs. saline; # $p < 0.05$ vs. nicotine.

Fig. 5. Effect of electro-acupuncture on perinatal nicotine-induced changes in lung myogenic proteins α -SMA and calponin

Compared with the control group, perinatal nicotine exposure resulted in increased α -SMA (5A) and calponin (5B) protein levels (Western analysis and immunostaining), with blockage of these changes with concomitant EA treatment. Representative lung sections for α -SMA (5A) and calponin (5B) immunostaining are shown. Values are mean $\pm$ SE; $n=6$ for each group. * $p < 0.05$ vs. saline; # $p < 0.05$ vs. nicotine.

Fig. 6. Effect of electro-acupuncture on perinatal nicotine-induced changes in key lung differentiation markers surfactant protein-A and -B

As determined by ELISA, compared with the control group, perinatal nicotine exposure resulted in an increase in whole lung SP-A (6A), but a decrease in whole lung SP-B (6B) levels; concomitant EA treatment blocked both of these changes. Values are mean $\pm$ SE; n=6 for each group. * $p < 0.05$ vs. saline; # $p < 0.05$ vs. nicotine.

Fig. 7. Effect of Electro-acupuncture on Perinatal Nicotine-Induced Changes in Key Markers of HPA axis

Compared with the control group, hypothalamic CRH levels (ELISA) in the nicotine-treated group decreased significantly; however, concomitant EA treatment blocked this decrease (Fig. 7A). In contrast, lung GR levels increased in the nicotine exposed group (Western and immunostaining), an effect that was normalized in the EA treated group (Fig. 7B and 7C). Representative lung sections for GR immunostaining are shown (7C). Values are mean $\pm$ SE; $n=6$ for each group. * $p < 0.05$ vs. saline; # $p < 0.05$ vs. nicotine.

Fig. 8. Effect of electro-acupuncture on perinatal nicotine-induced changes in circulating β-endorphin levels

As determined by ELISA, compared with the control group, perinatal nicotine exposure resulted in a significant decrease in plasma β-endorphin levels, which was blocked in the EA treated group. Values are mean ± SE; n=6 for each group. * $p < 0.05$ vs. saline; # $p < 0.05$ vs. nicotine.