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## Adrenergic and cholinergic responses of the tracheal smooth muscle is enhanced during estrus phase in rat

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**Aim:** The aim of this study was to evaluate influences of hormonal changes during rat estrous cycle on airway responsiveness to bronchoconstrictor and bronchodilator agents.

**Materials and methods:** The study was performed in 5 groups of rat tracheal smooth muscle obtained during proestrus, estrus, metestrus, and diestrus phases of the estrous cycle, as well as the ovariectomized group (n:8/group). Cumulative acetylcholine contractions ranging from  $10^{-8}$  to  $10^{-3}$  M bath doses and the cumulative isoproterenol relaxations ranging from  $10^{-8}$  to  $10^{-3}$  M precontracted with  $10^{-5}$  M of acetylcholine were recorded on isolated tracheas placed in a computerized tissue bath system.

**Results:** Acetylcholine-induced contractions ranging from  $10^{-5}$  to  $10^{-3}$  M were significantly enhanced dose-dependently in tracheal smooth muscle from estrus rats compared to those from metestrus and ovariectomized rats. The relaxation induced by isoproterenol ( $10^{-8}$ - $10^{-3}$  M) also significantly increased in tracheal smooth muscle precontracted with acetylcholine ( $10^{-5}$  M) taken from estrus rats. However, acetylcholine-induced contractility increase in tracheal smooth muscle was much more prominent than isoproterenol-induced relaxations in tracheal smooth muscle from estrus rats.

**Conclusion:** These findings indicated that changes in estrogen and/or progesterone levels during rat estrous cycle might affect acetylcholine and isoproterenol sensitivity of the tracheal smooth muscle.

**Key words:** Acetylcholine, estradiol, estrous cycle, isoproterenol, tracheal smooth muscle

### Sıçanda estrus fazı sırasında trakea düz kası adrenerjik ve kolinerjik cevapları artır

**Amaç:** Bu çalışmanın amacı sıçan estrus döngüsü sırasındaki hormonal değişikliklerin, bronkokonstriktör ve bronkodilatör ajanlara, solunum yolu hassasiyeti üzerine etkilerinin değerlendirilmesidir.

**Yöntem ve gereç:** Çalışma estrus döngüsünün proestrus, estrus, metestrus ve diestrus fazlarındaki ve ovariectomi uygulanmış sıçanlar olmak üzere 5 grup sıçandan (n:8/grup) elde edilen trakea düz kasında yapılmıştır. İzole trakealar bilgisayarlı organ banyosu sistemine yerleştirilip banyo dozları  $10^{-8}$  den  $10^{-3}$  M'a kadar olan kümülatif asetilkolin kasılmaları ve  $10^{-5}$  M asetilkolinle kastırılmış prekontrakte preparatlarda aynı dozajlardaki kümülatif izoproterenol gevşemeleri kaydedilmiştir.

**Bulgular:** Asetilkolinle ( $10^{-8}$ - $10^{-3}$  M) uyarılmış kasılmalar estrustaki sıçanlarda metestrustaki ve ovariectomi uygulanmış sıçanlarla karşılaştırıldığında doza bağlı olarak anlamlı bir şekilde artmıştır. Estrustaki sıçanlardan alınmış olan ve asetilkolinle önceden kastırılmış trakea düz kaslarında izoproterenolle ( $10^{-8}$ - $10^{-3}$  M) uyarılmış gevşemeler de anlamlı bir şekilde artmıştır. Ancak estrustaki sıçanlardan alınan trakea düz kasındaki asetilkolinle uyarılan kasılabilirlikteki artış izoproterenolle uyarılmış gevşemeden daha belirgindir.

**Sonuç:** Bu bulgular sıçan estrus döngüsü sırasındaki estrogen ve/veya progesteron seviyelerindeki değişikliklerin trakea düz kasının asetilkolin ve izoproterenole hassasiyetini etkilediğini göstermektedir.

**Anahtar sözcükler:** Asetilkolin, estradiol, estrus döngüsü, izoproterenol, trakea düz kası

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## Introduction

Hormonal changes during menstrual cycle in human and estrous cycle in animals may differentially influence physiologic and pathophysiological responses of body systems (1). Autonomic nerve function may be modulated in various ways, such as neurotransmitters, inflammatory mediators, or hormones. There are some reports emphasizing the complexity of interactions of factors that regulate smooth muscle response to adrenergic and cholinergic response differences depending on the phase of the estrous cycle (2-4). Receptor density of neurotransmitters in various target tissues is modulated by sex hormones, as well.

Evidence from several studies suggests a role for sex hormones in the pathogenesis of asthma. Among the general population, asthma prevalence is higher in women than in men (5). Female reproductive processes, such as pregnancy and menstruation, affect asthma, indicating a major role for the female sex hormones. Studies have suggested that respiratory function is influenced by female sex hormones and menstrual cycle phase (6,7). The lung has hormone receptors for both estrogen and progesterone (8-10). Gonzalez-Arenas et al. (11) reported that during the estrous cycle, various progesterone and estrogen receptors have been found at rat lung, among which progesterone receptor isoforms are the highest during proestrus and the lowest during estrus.

Since the sex steroid changes during the menstrual cycle differentially affect the smooth muscle sensitivity to cholinergic and adrenergic agents, changes in the tracheal smooth muscle sensitivity depending on the phase of estrous cycle may have important implications on the asthma profile of pregnant women and female patients, especially those who are given high doses of estrogen replacement therapy. However, observations regarding the influence of estrogens on asthma are conflicting. In a cohort study, long-term use and/or high doses of postmenopausal estrogen therapy have been reported to increase the subsequent risk of asthma (12). In contrast, others have suggested that estrogen treatment can have a beneficial effect on airway responsiveness (13) and on asthma (14). Degano et al.

(15) have shown that low physiologic doses of  $17\beta$ -estradiol given to ovariectomized female rats decreased airway responsiveness to acetylcholine. Effects of estrogen on whether muscarinic or adrenergic sensitivity of rat isolated tracheal smooth muscle is not clear. Because of this, in this study, we aimed to investigate effects of phases of rat sexual cycle on rat tracheal smooth muscle contraction to acetylcholine and relaxation to isoproterenol.

## Materials and methods

### Animals

Adult female Sprague-Dawley rats weighing 200-250 g obtained from animal house of our department were used for the study. Rats were housed under temperature-controlled room with a 12 h light: 12 h dark cycle (lights on at 7:00 am), and fed with standard pellet and water ad libitum. Animal studies were performed with the approval of Animal Care and Ethics Committee of Medical Faculty of Eskisehir Osmangazi University.

### Determination of estrous cycle

Estrous cycle phases were determined by daily vaginal smears every morning at 9:00 as described by Marcondes et al. (16) according to the following criteria: a proestrus smear consisting of a predominance of nucleated epithelial cells; an estrus smear primarily consisting of anucleated cornified cells; a metestrus smear consisting of the same proportion among leukocytes and cornified and nucleated epithelial cells; and a diestrus smear primarily consisting of a predominance of leukocytes. Only those rats that had at least 2 regular 4-day estrous cycles were used for the experiments.

### Ovariectomy

In one group, rats were bilaterally ovariectomized. Ovariectomies were performed under aseptic conditions. Animals were anesthetized with a mixture of xylazine hydrochloride (10 mg/kg, i.p.) and ketamine hydrochloride (25 mg/kg, i.p.). Dorsolateral incisions were made. The ovary and oviduct and a small section of the uterus were removed. Ovariectomies were performed at least 4 weeks before organ-bath studies.

### Organ-bath studies

After determination of the estrous cycle phase, the rats were sacrificed by cervical dislocation. Trachea between larynx and bifurcation was dissected out quickly and the connective tissues were gently removed. The trachea was cut into helical fashion in a cold oxygenated Krebs-Henseleit solution (pH 7.40) containing (in mM) NaCl (118), KCl (5.4),  $MgSO_4$  (1.2),  $KH_2PO_4$  (1.2),  $NaHCO_3$  (25.0), glucose (11.7), and  $CaCl_2$  (2.5). Each preparation was vertically mounted in a 10 mL organ-bath containing Krebs-Henseleit solution (pH 7.4, 37 °C) continuously bubbled with 95%  $O_2$  and 5%  $CO_2$ . The lower hooks were anchored to the bottom of the baths, and the upper hooks were attached to a force displacement transducer (Biopac, USA) connected to a data acquisition and analysis system (MP 100, Biopac, USA).

The preparations were allowed to equilibrate for 1 h under 1 g initial tension. During the equilibration period, the bath solution was changed every 15 min. After the equilibrium period, the trachea was tested with 80 mM KCl to evaluate the viability of the tissue.

The preparations were exposed to acetylcholine ( $10^{-8}$ - $10^{-3}$  M, Sigma-Aldrich, USA) or isoproterenol ( $10^{-8}$ - $10^{-3}$  M, Sigma-Aldrich, USA). Concentrations were not increased until the response to the previous concentration had stabilized. For isoproterenol-induced relaxation studies following the equilibration period, trachea was precontracted with  $10^{-5}$  M acetylcholine.

### Statistical analysis

All data were expressed as mean  $\pm$  SD. Statistical differences of the results were determined by one-way analysis of variance followed by a Tukey test. Statistical significance was considered as  $P < 0.05$ .

### Results

Acetylcholine treatment to tracheal preparations produced dose dependent contraction as shown in Figure 1. With  $10^{-5}$  M to  $10^{-3}$  M doses, acetylcholine-induced contractions in estrus (maximal contraction;

$502.5 \pm 124$  mg) were significantly greater than those in metestrus (maximal contraction;  $331.8 \pm 25$  mg) and ovariectomized (maximal contraction;  $355.4 \pm 113$  mg) groups ( $P < 0.05$ ) (Figure 1).

Isoproterenol-induced relaxations in the trachea precontracted with acetylcholine were significantly enhanced in estrus (maximal relaxation;  $112.1 \pm 15$  mg) when compared with proestrus (maximal relaxation;  $46.3 \pm 10$  mg) and diestrus (maximal relaxation;  $72.9 \pm 23$  mg) as illustrated in Figure 2 ( $P < 0.05$ ).

Contraction sensitivity was much more prominent than relaxation sensitivity of tracheal smooth muscle in estrus;  $EC_{50}$  of acetylcholine-induced contraction increased to  $236.20 \pm 59$  mg, but  $EC_{50}$  of isoproterenol-induced relaxation was only  $72.65 \pm 9.25$  mg.

### Discussion

Involvement of estrogens on asthma development has not been clearly defined yet. Estrogen treatments

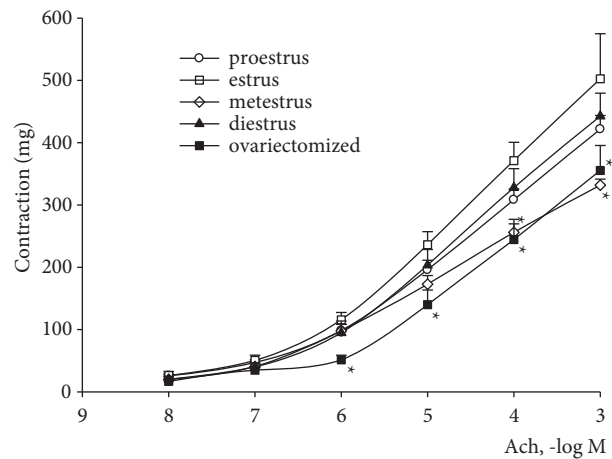


Figure 1. Cumulative contraction response to acetylcholine in tracheal smooth muscle from different phases of rat estrous cycle: proestrus (n = 8), estrus (n = 8), metestrus (n = 7), diestrus (n = 8), and ovariectomized (n = 8). Data were expressed as mean  $\pm$  SD of absolute force (mg) values. \* $P < 0.05$  compared with the estrus data.

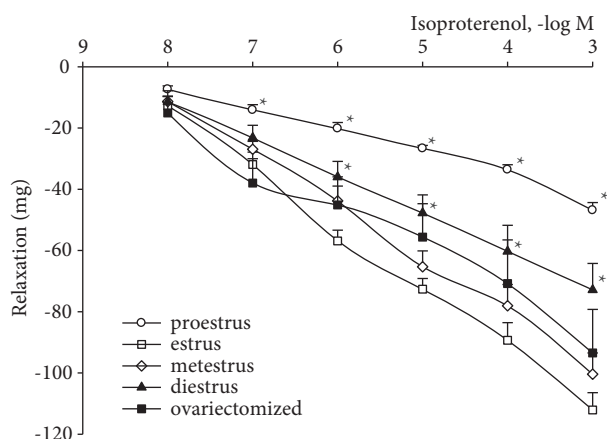


Figure 2. Cumulative relaxation response to isoproterenol in tracheal smooth muscle precontracted with acetylcholine ( $10^{-5}$  M) from different phases of rat estrous cycle: proestrus ( $n = 8$ ), estrus ( $n = 8$ ), metestrus ( $n = 7$ ), diestrus ( $n = 8$ ), and ovariectomized ( $n = 8$ ). Data were expressed as mean  $\pm$  SD of absolute force (mg) values. \* $P < 0.05$  compared with the estrus data.

have been reported either to be beneficial on airway responsiveness (12) or to increase the risk of asthma (13,14). The present study suggested that changing sex steroid profile during estrous cycle modulates cholinergic and adrenergic responses of isolated rat tracheal smooth muscle.

At estrous cycle, estrogen is high prior to the ovulation and is low following the ovulation (17-19). Herein we show that cholinergic contraction of tracheal smooth muscle is greater at high estrogen levels than at low estrogen levels. Consistent with this observation, Degano et al. (20) reported that in ovariectomized female rats, chronic treatment with  $17\beta$ -estradiol could alter airway reactivity in a dose-dependent manner: low dose estradiol treatment decreases and high dose estradiol treatment increases the potency of acetylcholine.

In the present study, we showed that cholinergic contraction and adrenergic relaxation of tracheal smooth muscle were highest in estrus. Consistent with this observation, Honda et al. (3) reported that acetylcholine-induced relaxation of the thoracic aorta was greatest in estrus.

Medlock et al. (21) showed that physiologic doses of  $17\beta$ -estradiol elevate uterus nuclear estrogen receptors within 1-3 h; indicating that estrogens stimulate protein synthesis in hours. Klangkalya et al. (4) reported that cardiac muscarinic and beta-adrenergic receptor density increased upon treatment with progesterone and estrogen together in ovariectomized rats. Moreover, Levin et al. (22) reported that density of muscarinic and adrenergic receptors increased in rabbit bladder given 4-days of estradiol treatment. Additionally, Wilkinson et al. (23) reported that estrogen induces an increase in sensitivity of alpha-receptors and numbers of beta-receptors in rat hypothalamus. Thus, we suggested that one of the reasons why both acetylcholine induced contraction and isoproterenol induced adrenergic relaxation were highest in estrus that following proestrus may be the result of time dependent genomic effects of the elevated estrogen during proestrus. In other words, the effects of the elevated estrogen in proestrus may change acetylcholine and isoproterenol sensitive receptor activity and density of the tracheal smooth muscle of the rat.

Our study suggested that the lowest contraction to acetylcholine was seen in metestrus and ovariectomized rats that were expected to have the lowest estrogen concentration, which further supports the idea that increased estrogen may affect acetylcholine and isoproterenol sensitive receptor activity of tracheal smooth muscle. Additionally, the presence of high progesterone that hyperpolarizes smooth muscles during metestrus may also be involved in the relaxation response and the suppressed contraction response. On the other hand, there are 2 progesterone peaks: one in metestrus and the other in proestrus (17,19). The elevated progesterone during proestrus may counteract on the acetylcholine induced contractions in this phase, as well.

Considering the reports cited above and our results, increasing acetylcholine-induced contraction and isoproterenol-induced relaxation in tracheal smooth muscle during estrus phase may be due to; i. the increase in production of estrogens and/or

progesterone dependent bronchodilators and bronchoconstrictors, ii. changes in adrenergic and/or cholinergic receptor sensitivity and density, iii. changes in adrenergic and cholinergic metabolizing systems, iv. direct-indirect potentiation of adrenergic and cholinergic responses, and v. estrogen induced modulation of intracellular  $Ca^{2+}$  mobilization.

In conclusion, our data support the idea that changes in steroid profile during estrous cycle alter airway smooth muscle contraction and relaxation

responses. The increased contractile response of the tracheal smooth muscle to acetylcholine that was more prominent than the relaxation response to isoproterenol might implicate that those acetylcholine-induced contractions might be more sensitive to estrogens than isoproterenol-induced relaxations. This sensitivity may have important implications for the asthma profile of female patients especially who are given high doses of estrogen replacement therapy.

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