Eur Arch Otorhinolaryngol (2012) 269:1945–1950
DOI 10.1007/s00405-012-1978-7

LARYNGOLOGY

Histological evaluation of rat larynx in experimental polycystic ovary syndrome model

Hande Senem Deveci · Ildem Deveci · Mehmet Habesoglu · Mehmet Sürmeli · Vefa Kınıs · Murat Eriman · Pembegul Gunes · Murat Yekrek · Erol Egeli

Received: 3 November 2011 / Accepted: 17 February 2012 / Published online: 11 March 2012

Abstract This study aimed at studying the histopathological effects of hyperandrogenemia and estrogen deficiency on larynx mucosa in experimentally designed polycystic ovary syndrome of female rats. Two groups of experimental polycystic ovary syndrome model were composed in healthy female rats by per oral letrozole administration of for 21 and 42 days. Also a control group which only took vehicle (saline) for 42 days was designed. Laryngeal mucosa and ovaries of all animals were examined histopathologically by light microscopy and the serum hormone levels were analyzed using a solid-phase, two-site chemiluminescent enzyme immunometric assay. Statistically significant edema, vascular engorgement, inflammation, cilia loss and differentiation of goblet cell distribution were observed when the control group and study groups were compared ($p < 0.01$). In serum hormonal analysis there was a significant increase in levels of androgens and decrease in levels of estrogens. In addition, polycystic appearance of ovaries in letrozole-administered groups and normal appearance of ovaries in control group have been proven histopathologically. Polycystic ovary syndrome which causes estrogen deficiency and hyperandrogenemia in fertile ages resulted in histopathological changes in laryngeal mucosa.

Keywords Larynx · Hyperandrogenism · Histopathology

The study was done in Haydarpaşa Numune Education and Research Hospital.

H. S. Deveci (✉)
Department of Otolaryngology-Head and Neck Surgery,
Fatih Sultan Mehmet Education and Research Hospital,
E-5 üzeri Bostancı, 34744 Istanbul, Turkey
e-mail: deveci2006@yahoo.com

I. Deveci
Department of Otolaryngology-Head and Neck Surgery,
Pasabahce Goverment Hospital, Istanbul, Turkey
e-mail: ildemdeveci78@yahoo.com

M. Habesoglu
Department of Otolaryngology-Head and Neck Surgery,
Umraniye Education and Research Hospital, Istanbul, Turkey
e-mail: mhabesoglu@yahoo.com

M. Sürmeli
Department of Otolaryngology-Head and Neck Surgery,
Karaman Goverment Hospital, Karaman, Turkey
e-mail: drsurmelimehmet@hotmail.com

V. Kınıs
Department of Otolaryngology-Head and Neck Surgery,
Diyarbakir Dicle University Hospital, Diyarbakır, Turkey
e-mail: vefakinis@hotmail.com

M. Eriman
Department of Otolaryngology-Head and Neck Surgery,
Diyarbakir Ergani Goverment Hospital, Diyarbakır, Turkey
e-mail: murateriman2001@mynet.com

P. Gunes
Department of Pathology, Haydarpasa Numune Education
and Research Hospital, Istanbul, Turkey
e-mail: pembegulgunes@gmail.com

M. Yekrek
Department of Medical Biochemistry, Haydarpasa Numune
Education and Research Hospital, Istanbul, Turkey
e-mail: mmuratyekrek@yahoo.com

E. Egeli
Department of Otolaryngology-Head and Neck Surgery,
Haydarpasa Numune Education and Research Hospital,
Istanbul, Turkey
e-mail: erolegeli@rinodentistanbul.com

Introduction

Larynx is very sensitive to endocrinologic changes. Larynx, endometrium, prostate and breast are accepted as secondary sex organs. Clinical studies suggest that premenopausal and postmenopausal changes in gonadal function modify auditory [1–3], taste [4] and olfactory [4–7] thresholds, larynx [8] and nasal mucosa [9, 10]. In recent studies altered voice quality in menopause is assumed to be due to estrogen deficiency and relative increase in androgen levels. [11, 12]. In another study, the impact of menopause on the female rat larynx in ovariectomized animals was presented histopathologically [13]. Just like menopause, polycystic ovary syndrome (PCOS) also modifies hormone profile in women. PCOS affects 5–8% of the fertile-age women worldwide [14, 15]. This syndrome is characterized by chronic anovulation and hyperandrogenism, which is a common endocrine abnormality that affects women in fertile age. Infertility, abortus, hirsutism, obesity, metabolic syndrome, increased gestational diabetes risk and increased cardiovascular disease risk are the reported complications of PCOS. However, none of the studies about PCOS has emphasized the histopathological evidence of otorhinolaryngological pathologies.

There are different methods to compose experimental PCOS model in the laboratory rat such as exposure to continuous light, developing anterior hypothalamic and amygdaloidal lesions, administration of androgens, administration of estrogens to rats during early postnatal life and giving antiprogestins or using aromatase inhibitors [16]. We preferred aromatase inhibitors for developing PCOS model. As an aromatase inhibitor we used letrozole because of its easy daily single-dose use, being cheaper than gonodotropins and having many similarities in serum hormonal panel of PCOS [17–19].

In this study, our aim was to evaluate the histopathological changes of laryngeal mucosa of female rats with PCOS.

Methods

Study design and setting

Eight-week-old healthy female Albino Wistar rats (mean body weight 221,05 ± 11.1gr) with regular estrus cycles with duration of 4–5 days were maintained from Haydarpasa Numune Education and Research Hospital experimental animal laboratory. To prove regularity of estrus cycles, we monitored vaginal smears daily for three consecutive cycles before drug administration. All animals were kept in 22 ± 2°C temperature, were fed with standard laboratory foot and were exposed to 12-h light, 12-h darkness. All experiments were carried out with the approval of the Haydarpasa Numune Education and Research Hospital Animal Use Committee and animal studies was done only by the researcher who has experimental animal study certificate in accordance with the Environment and Forestry Ministry's animal protection law.

Table 1 Distribution of groups

Groups	*n*	Given treatment	Treatment time (days)
Group I	*n* = 6	1 mL saline p.o. (once daily)	42
Group II	*n* = 10	1 mg/kg letrozol + 1 mL saline p.o. (once daily)	21
Group III	*n* = 10	1 mg/kg letrozol + 1 mL saline p.o. (once daily)	42

The animals were divided into three groups, including a control group of six rats that received per oral vehicle (1 mL saline) once daily for 42 days and two study groups of ten rats each treated with per oral letrozole (Femara®, Novartis, Istanbul, Turkey) dissolved in 1 mL saline once daily at concentrations of 1 mg/kg; for each study group, letroze was administered for 21 days and 42 days, respectively (Table 1).

Hormone analysis

24 h after the last dose of letrozole and saline administration all rats were anesthetized with ketamin hydrochloride (66 mg/kg ip. and 44 mg/kg im) and decapitated. Before decapitation of rats, intracardiac blood samples were obtained for assessment of serum testosterone and estradiol levels. Samples were analyzed using a solid-phase, two-site chemiluminescent enzyme immunometric assay (Immunlite; EURO/DPC, Llaberies, UK).

Tissue sampling

After obtaining intracardiac blood samples, the rats were dissected and the larynges and ovaries were removed without disturbing the integrity of the laryngeal mucosa. Samples were immediately fixed in 10% formaldehyde solution. They were embedded in paraffin blocks. Larynx specimens were sliced to 5-μm thickness, whereas ovary specimens were sliced to 10-μm thickness. All slices were stained with Hemotoxylin–Eosin and examined by light microscope at a magnification of 10×, 40×, and 60× to compare the findings among the three groups. Histopathological changes of larynx specimens were considered as edema, vascular engorgement, inflammation, cilia loss and goblet cell distribution. Ovary specimens were examined to prove the polycystic appearance of the ovaries in study groups and normal appearance of ovaries in control group.

All slices were coded to avoid observer's bias during examination and analyzed by one pathologist. Pathologist graded the larynx specimens to create a semi-quantitative histology scoring.

To evaluate edema and vascular engorgement in light microscopy, a score system was designed according to the degree of edema or vascular engorgement degree: grade 0 (none), grade 1 (mild), grade 2 (moderate) or grade 3 (marked). To qualitatively evaluate the severity of inflammation, infiltration of polymorphonuclear leucocytes (PMNL) and lymphocytes was taken into account. They were graded as 0 (none; infiltration of 1–20 lymphocytes, no PMNL), 1 (mild; infiltration of 21–50 lymphocytes, 1–2 PMNLs), 2 (moderate; 51–80 lymphocytes, 3–10 PMNLs), 3 (severe; infiltration of 81–120 lymphocytes, >10 PMNLs). Cilia loss was examined morphologically by light microscopy. When cilia were observed lesser than normal in areas where they should be normally seen, it was graded as mild [1]. If there were severe loss of cilia, it was graded as moderate [2]; and if total loss was observed, it was graded as severe [3]. The number of goblet cells visible in microscopy was counted.

Statistical analysis

All statistical calculations were performed with NCSS 2007 and PASS 2008 Statistical Software (NCSS, Kaysville, UT). Furthermore, standard descriptive statistical calculations (mean, median and standard deviation), quantitative comparison of the data and the comparison of normal distribution parameters of the groups were assessed with Oneway Anova test, and the Kruskal–Wallis test was used in the assessment of not normal distributed parameters according to groups and the Mann–Whitney *U* test was used for the evaluation of differences. The statistical significance level was established at $P < 0.05$, and confidence intervals were 95 percent.

Results

Serum estradiol and testosterone levels

The mean serum estradiol (E2) levels were 62.0 ± 16.28 in control group and 24.7 ± 10.44; 18.20 ± 5.30 in 21- and 42-day groups, respectively. The difference of mean serum E2 levels between the control group and 21-day study group was not statistically significant ($p > 0.508$) but the difference of mean serum E2 levels between the control group and 42-day group was statistically significant ($p < 0.05$). The mean serum testosterone levels were 117.93 ± 42.20 in control group and 703.46 ± 446.97; 792.90 ± 576.64 in 21- and 42-day groups, respectively. The differences of mean serum testosterone levels between the control group and 21-, 42-day groups were statistically significant, respectively ($p < 0.05$).

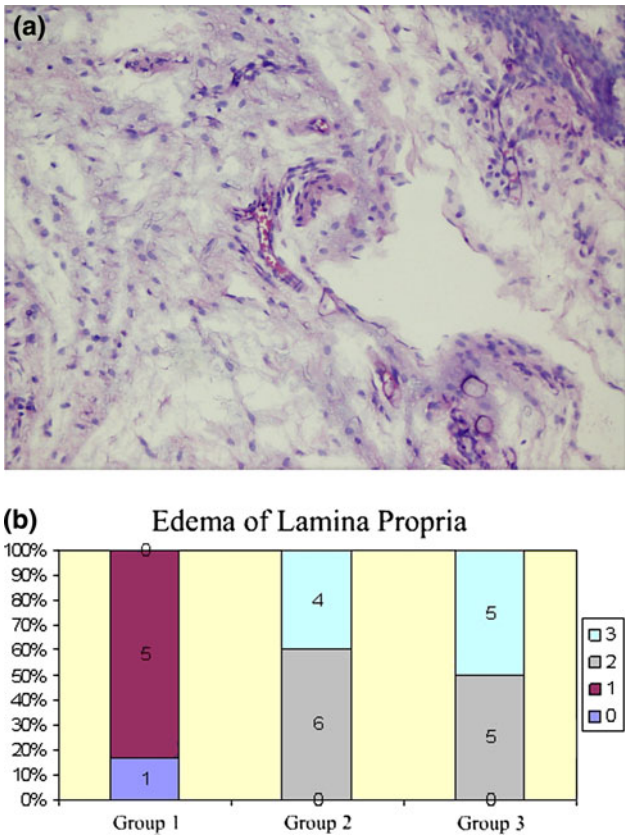

Fig. 1 **a** Edema of lamina propria (grade 3), (HE&E 60 × 10). **b** Distribution of degree of edema of lamina propria

Edema

When we assessed edema of lamina propria between the control group and the study groups, there were significant difference ($p < 0.01$).The assessment of edema of lamina propria between the control group and 21-, 42-day study groups revealed significant differences, respectively ($p = 0.001$; $p = 0.001$; $p < 0.01$) (Fig. 1a, b).

Vascular engorgement

The assessment of vascular engorgement between the control group and the study groups presented significant difference ($p < 0.01$). The comparison of vascular engorgement between the control group and 21-, 42-day treatment groups revealed significant differences, respectively ($p = 0.001$; $p = 0.001$; $p < 0.01$) (Fig. 2a, b).

Inflammation

When we examined the specimens in light microscope, we observed significant inflammation between the control group and the study groups revealed significant difference

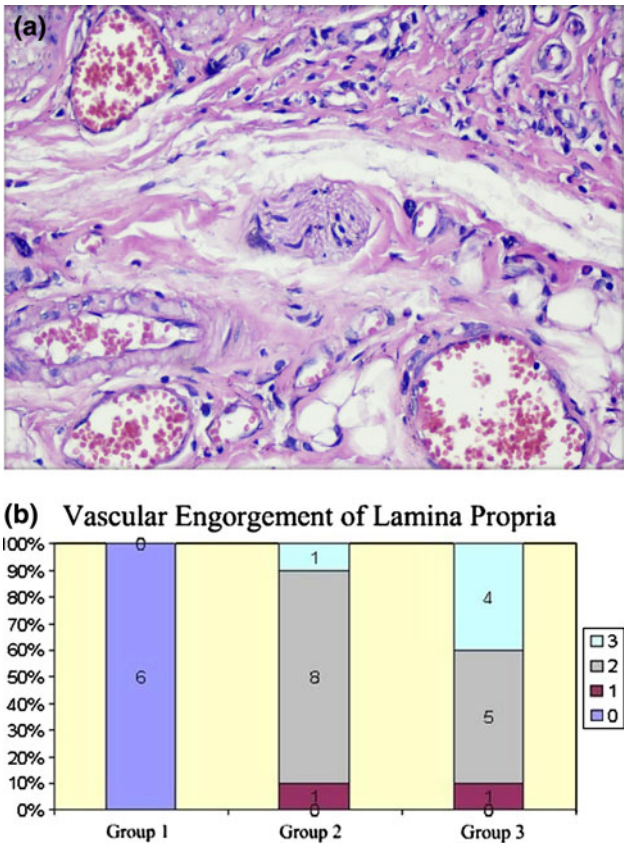

Fig. 2 **a** Vascular engorgement of lamina propria (grade 2), (HE&E 60 × 10). **b** Distribution of degree of vascular engorgement of lamina propria

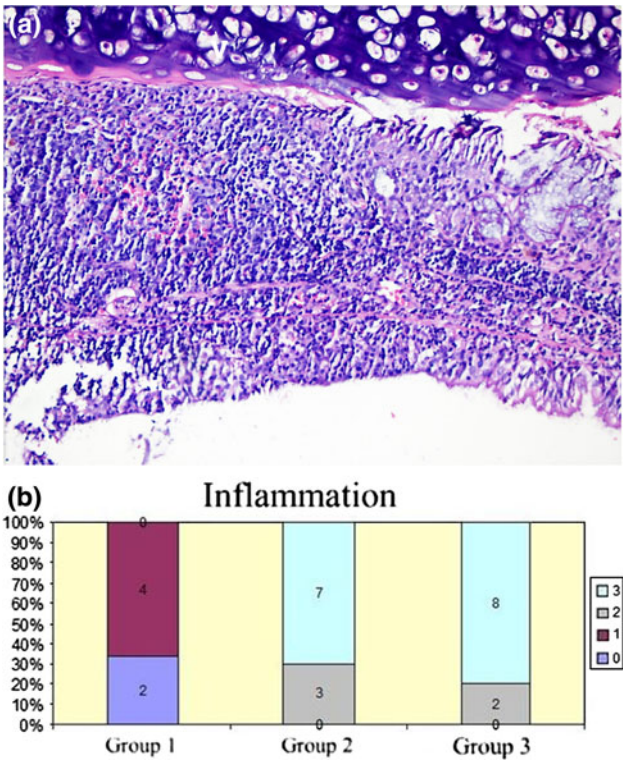

Fig. 3 **a** Inflamation (grade 3), (HE&E 60 × 10). **b** Distribution of degree of inflamation

($p < 0.01$). The comparison of inflammation between the control group and the 21-, 42-day study groups revealed significant differences, respectively ($p = 0.001$; $p = 0.001$; $p < 0.01$) (Fig. 3a, b).

Cilia loss

The evaluation of cilia loss between the control group and study groups displayed significant difference ($p < 0.01$). The comparison of cilia loss between the control group and 21-, 42-day study groups revealed significant differences, respectively ($p = 0.001$; $p = 0.001$; $p < 0.01$), (Fig. 4a, b).

Goblet cell distribution

When we assessed goblet cell distribution between the control group and study groups, there were significant difference ($p < 0.01$). The comparison of goblet cell loss between control group and 21-day group revealed no significant difference ($p > 0.05$), whereas the control group and 42-day treatment group comparison revealed significant difference ($p = 0.016$; $p < 0.05$) (Table 2).

Table 2 Comparsion of goblet cell distribution between control and study groups

	Goblet cell number		p^a
	Mean ± SD	Median	
Group I ($n = 6$)	7.16 ± 2.92	6.5	0.001**
Group II ($n = 10$)	7.60 ± 5.52	4.0	
Group III ($n = 10$)	4.0 ± 4.02	3.0	
Group I–II	0.508		
Group I–III	0.016*		
Group II–III	0.026*		

* $p < 0.05$, ** $p < 0.01$

[a] Kruskall–Wallis test

Discussion

During the lifetime, the larynx is very sensitive to fluctuations in the levels of sex hormones. Voice is affected by combination of aging and sex hormone levels in both male and female genders [20, 21]. In recent studies, sex hormone receptors have been detected in human respiratory tract and the hormonal impact on airway have been demonstrated [11, 12, 22]. Although there are studies about the impact of hormones on larynx, the whole mechanism of this effect has

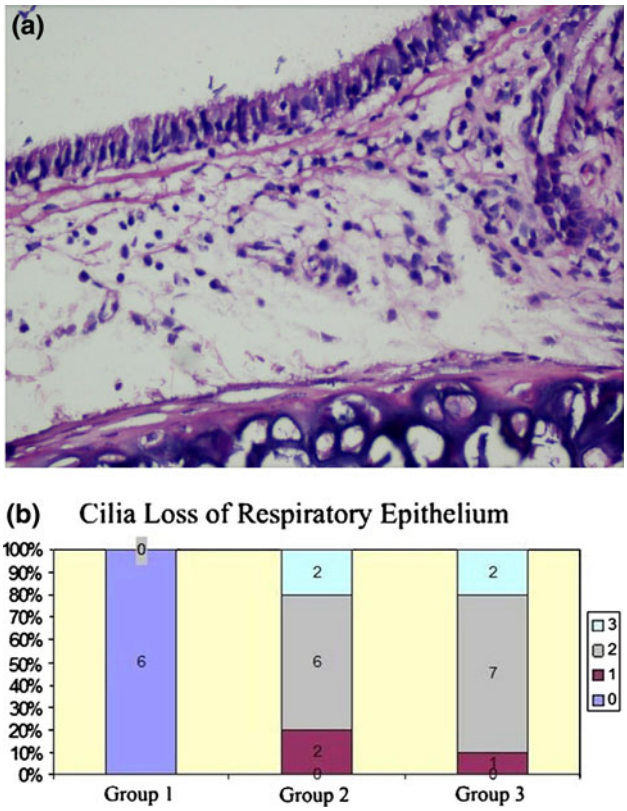

Fig. 4 **a** Cilia loss of respiratory epithelium (grade 2), (HE&E 60 × 10). **b** Distribution of severity of cilia loss

not been clear yet. Most of the studies search for receptors and found some receptors especially on the vocal cords. In our study, we have demonstrated that not only the vocal cords, but also the entire larynx was affected by altered hormone profile.

Comparisons of prepubertal, pubertal and adult female larynges showed that the female larynx reaches adult size by puberty [23]. Vocal tract size also increases significantly at puberty [24], although the alteration is more significant in boys than in girls. Laryngeal maturation caused by androgen stimulation after puberty is the main cause of difference in male and female voices, although the direct mechanism is not clearly understood.

Androgens cause water and salt intake in the body, induce protein synthesis and induce vascularisation. In our study when we compared study groups with control group, we detected statistically significant vascular engorgement, increased inflammation and edema in lamina propria. These findings could be due to increased androgen and decreased estrogen levels.

Impact mechanisms of hormones on larynx have been studied in different researches. Higgins and Saxman [25] described that the hormonal fluctuations impact the neurotransmitter levels which result in changes in motor and sensory process involved in laryngeal control.

Mund et al. had examined bronchoalveolar lavage (BAL) of middle-aged and elderly women with long-lasting dry cough that was resistant to all kinds of therapies. In this study, BAL fluid contained increased number of $CD4^+$ lymphocytes [26]. Their hypothesis was that enhanced capacity in T helper lymphocyte function in menopaused women might lead to exaggerated reactions to exposures such as infections. Interestingly in this study, also there was an increased coexistence of infertility, dysmenorrhea, ectopic pregnancy and miscarriage. This invention strengthens our findings. Also in our study, there was a lymphocyte dominate chronic inflammation in entire larynx. In addition, loss of cilia was increased in areas with intense inflammation. Our hypothesis about these findings is that changes contribute to commonly seen symptoms such as dysphagia, chronic dry cough and globus sensation. Further new researches are needed to prove the interference of hormonal levels and these common symptoms in humans.

Histologically, 8-week testosterone administration in female albino mice causes parakeratosis and squamous metaplasia of the epithelium, thyroaritenoid muscle fiber hypertrophy and hyperplasia of seromucinous glands irreversibly after [27]. In our study, we did not observe any squamous metaplasia.

Also in our study, we detected decreased Goblet cell number. This finding could be the cause of decreased and altered upper respiratory tract mucous viscosity and structure. Alteration in mucous structure could result in deterioration in cilia motility. Clinically, cilia motility distortion cause increase upper and lower respiratory tract infections, increase duration of healing period and distortion of microparticule clarifying function of airways.

Conclusion

PCOS affects 5–8% of the fertile-age women worldwide [14, 15]. Present results suggest that PCOS which cause hyperandrogenism and estrogen deficiency affect rat's entire larynx histopathologically. In developed countries due to increased life-quality, also life expectations of women have been improved. Therefore, with each passing day, more women have been referred to otorhinolaryngology clinics for globus sensation, dysphagia, dry cough and voice impairment. Although PCOS is a common disease, its otorhinolaryngological manifestations have not been studied by ear-nose-throat doctors enough. Further researches are needed to define the effects of PCOS in humans otorhinolaryngologically.

Conflict of interest We do not have any financial relationship with the organization that sponsored the research nor conflict of interest.

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