Eur Arch Otorhinolaryngol (2011) 268:261–266
DOI 10.1007/s00405-010-1337-5

LARYNGOLOGY

Histopathological effects of estrogen deficiency on larynx mucosa in ovariectomised rats

Mehmet Surmeli · Tulay Erden Habesoglu · Mehmet Habesoglu · Ildem Deveci · Murat Eriman · Vefa Kinis · Pembegul Gunes · Erol Egeli

Received: 1 February 2010 / Accepted: 5 July 2010 / Published online: 18 July 2010

Abstract Laryngeal manifestations due to estrogen deficiency have been studied in the literature. But to date, the possible histopathological changes of laryngeal mucosa due to estrogen deficiency have not been studied. Therefore, our objective was to determine the histopathological changes of laryngeal mucosa in ovariectomised rats in order to clarify effects of estrogen deficiency on laryngeal tissue. The study is a randomized trial and was conducted at the animal care facility of Haydarpasa Numune Education and Research Hospital. Twenty-one Wistar rats were used throughout the experiment. There were six rats in the sham-operated control group. And others were divided into two groups (4, 8 weeks) according to follow-up time after ovariectomy. We observed significant changes 4 weeks after ovariectomy when we assessed subepithelial edema, inflammation, cilia and goblet cell loss ($p < 0.01$). It was shown that estrogen deficiency after ovariectomy in rats caused changes in laryngeal tissue when it was studied histopathologically.

Keywords Larynx mucosal · Estrogen deficiency · Histopathology

M. Surmeli · T. E. Habesoglu (✉) · M. Habesoglu · I. Deveci · M. Eriman · V. Kinis · E. Egeli
Department of Otorhinolaryngology, Haydarpasa Numune Education and Research Hospital, Istanbul, Turkey
e-mail: tulayhabesoglu@yahoo.com

P. Gunes
Department of Pathology, Haydarpasa Numune Education and Research Hospital, Istanbul, Turkey

Introduction

During menopause the ovaries lose their ability to produce estrogens and to ovulate. As a result of estrogen deficiency, menopausal symptoms, such as hot flashes and decreased bone density occur. Also, ovarian dysfunction causes mucosal atrophy and edema in urogenital system [1].

Although in women urogenital system is the major target of estrogen, there are other tissues which have estrogen receptors, so that they also become target organs of estrogen [2]. There exists some evidence of a probable modulating effect of sex steroids on the larynx [3–5].

Recently, some studies on laryngeal manifestations due to estrogen deficiency have been done [6, 7]; among them studies especially about vocal quality has come to the fore [8]. However, none of them emphasizes the histopathological evidence of laryngeal pathologies related to estrogen deficiency.

Thus, the goal of our study was to investigate the histopathological changes of laryngeal mucosa in ovariectomised rats in order to clarify effects of estrogen deficiency on laryngeal tissue.

Materials and methods

Study design and setting

Young adult female Wistar rats (9–10 months old) weighing 210–220 g were housed in stainless-steel mesh cages in a temperature controlled room (25°C) on a 12-h light, 12-h dark cycle. The animals were maintained ad libitum on water and rodent laboratory chow. All experiments were carried out with the approval of the Haydarpaşa Numune Education and Research Hospital

animal use committee. Efforts were made to minimize animal suffering and to reduce the number of animals used.

The animals were divided into groups according to follow-up time after ovariectomy.

Group 1 Sham-operated control group ($n = 6$).
Group 2 Surgically ovariectomised rats which were followed up a period of 4 weeks ($n = 7$).
Group 3 Surgically ovariectomised rats which were followed up for 8 weeks ($n = 8$).

Surgical procedure

In order to achieve estrogen deficiency in rats, ovariectomy operations were performed. Ovariectomies were carried out under ketamine HCL anesthesia (35 mg/kg, im). The fur on both sides of the body was shaved from hip to the lowest rib. Bilateral ovariectomies were performed using an incision 1.5 cm inferior to palpated rib cage. Ovaries and surrounding fat tissue were removed; the incision was closed by suturing the muscles and skin. Similar surgical procedures were carried out for the sham-operated animals except that the ovaries were not removed.

After at least 2 weeks of recovery rats were divided into their groups. At the end of their follow-up periods, rats were anesthetized with ketamine HCl (66 mg/kg ip and 44 mg/kg im) and decapitated. Their larynges were dissected and laryngeal mucosa were removed without disturbing their integrity.

Hormone analysis

Before decapitation of rats, intracardiac blood samples for assessment of serum hormone levels were obtained. Blood samples were analyzed by using a solid-phase two-site chemiluminescent enzyme immunometric assay. Immulite; EURO/DPC, Llanberis, United Kingdom (estrogen pg/ml).

Pathologic evaluation

Specimens were put in 10% formaldehyde solution. Specimens were sliced to 10 μm thickness and stained with hematoxylin and eosin. All of the slices were examined in blinded fashion by our pathologist under light microscopy to identify histopathological changes. The histopathological changes were considered as edema, vascular dilatation, inflammation in lamina propria and inflammation, cilia loss, goblet cell number in respiratory epithelium.

To evaluate subepithelial edema magnification of 100, a score was assigned according to the degree of them: 0 (none), 1 (mild), 2 (moderate), or 3 (marked).

To qualitatively evaluate the severity of inflammation, hyperplasia of the epidermis, amount of exudate on the epithelial surface, extent of thickening of the dermis, blood and lymph vessel dilation and infiltration of polymorphonuclear leucocytes (PMNL) and lymphocytes were taken into account. They were graded as +, mild inflammation (infiltration of few PMNL and lymphocytes); ++, moderate inflammation (infiltration of some PMNL and lymphocytes); +++, severe inflammation (severe infiltration of many PMNL and lymphocytes).

Cilia loss was considered morphologically by light microscopy. When cilia were observed lesser than normal in areas where they should be normally seen, it was graded as mild (+). If there were severe loss of cilia, it was graded as moderate (++); and if total loss was observed, it was graded as severe (+++).

At a magnification of 400, the number of goblet cells visible were counted.

Statistical analysis

All statistical calculations were performed with NCSS 2007 and PASS 2008 Statistical Software (Utah, USA). Besides standard descriptive statistical calculations (mean, median and standard deviation), Kruskal–Wallis test was used in the assessment of parameters according to groups, Mann–Whitney U test was used for the evaluation of differences. The statistical significance level was established at $p < 0.05$ and confidence interval was 95%.

Results

Serum estrogen level

The mean serum estrogen levels were 78.04 ± 8.82 in control group and 51.27 ± 5.36; 34.41 ± 7.16 in 4 week and 8 week groups, respectively. The differences of mean serum estrogen levels between the groups were statistically significant ($p < 0.01$).

Subepithelial edema

When we assessed submucous gland hyperplasia between the control group and the study groups, there were significant difference ($p < 0.05$). The assessment of subepithelial edema between the control group and 4-, 8-week study groups revealed significant differences, respectively ($p = 0.001$; $p = 0.001$; $p < 0.01$) (Fig. 1a, b) (Table 1).

Inflammation

The assessment of inflammation between the control group and the study groups revealed significant difference ($p < 0.05$). The comparison of inflammation between the

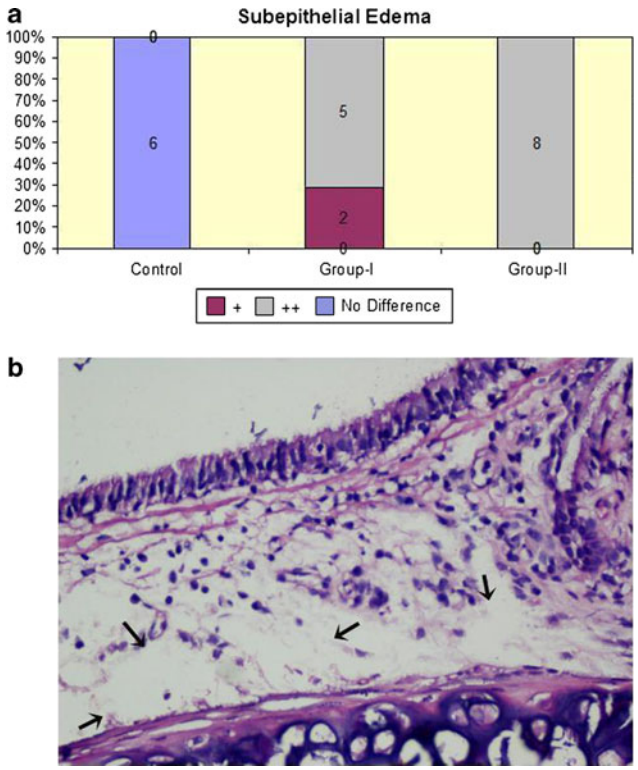

Fig. 1 **a** Subepithelial edema distribution of the groups. **b** Subepithelial edema in larynx mucosa (HE&E 60 × 10)

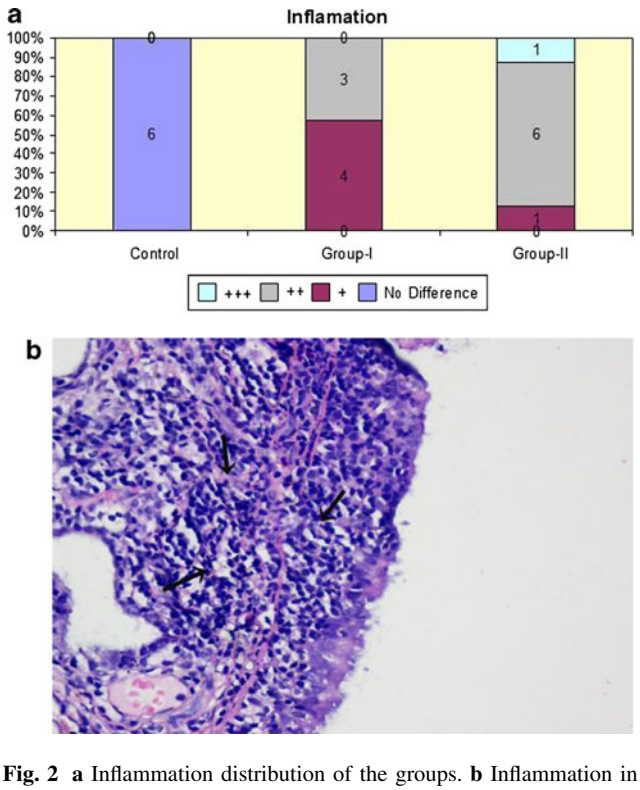

Fig. 2 **a** Inflammation distribution of the groups. **b** Inflammation in larynx mucosa (HE&E 60 × 10)

Table 1 Comparison of changes in larynx

	Subepithelial edema	Inflammation	Cilia loss
Control group I	0.001**	0.001**	0.013*
Control group II	0.001**	0.001**	0.001**

Mann–Whitney *U* test was used to evaluate post hoc subgroup analysis

$p < 0.05$; ** $p < 0.01$

control group and 4-, 8-week groups revealed significant differences, respectively ($p = 0.001$; $p = 0.001$; $p < 0.01$), (Fig. 2a, b) (Table 1).

Cilia loss

When we assessed cilia loss between the control group and the study groups, there were significant difference ($p < 0.05$). The comparison of cilia loss between the control group and 4-, 8-week groups revealed significant differences, respectively ($p = 0.013$; $p = 0.001$), (Fig. 3a, b) (Table 1).

Goblet cell distribution

When we examined laryngeal tissue specimens in light microscope, we observed significant goblet cell loss between control and study groups, respectively ($p = 0.001$; $p = 0.001$; $p < 0.01$), (Fig. 4; Table 2).

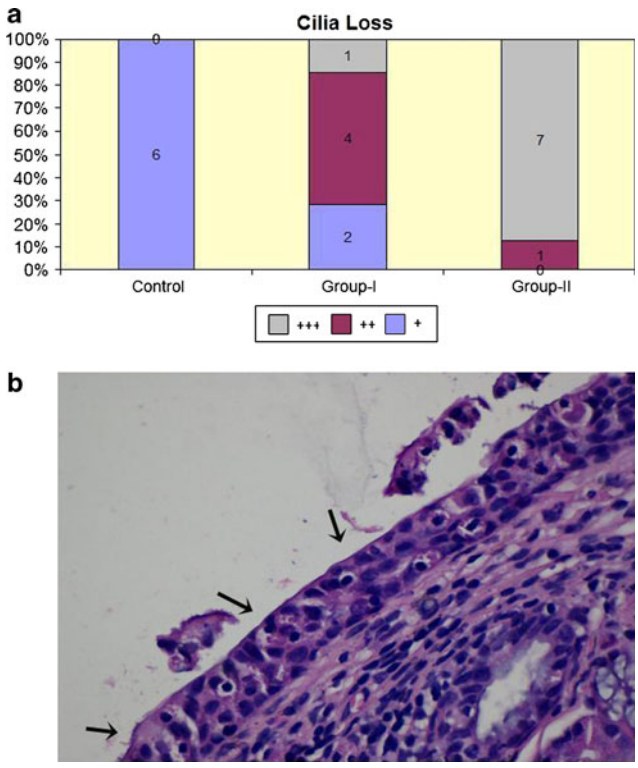

Fig. 3 **a** Cilia loss distribution of the groups. **b** Cilia loss in larynx epithelium (HE&E 60 × 10)

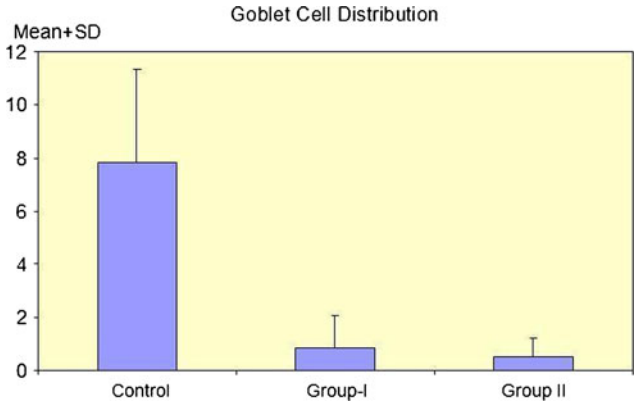

Fig. 4 Goblet cell distribution of the groups

Table 2 Comparison of Goblet cell distribution between control and study groups

	Goblet cell distribution		p^a
	Ort ± SD	Median	
Control ($n = 6$)	7.83 ± 3.54	7.5	0.001**
Group I ($n = 7$)	0.85 ± 1.21	0	
Group II ($n = 8$)	0.50 ± 0.75	0	
Control group I	0.001**		
Control group II	0.001**		

[a] Kruskal–Wallis test

** $p < 0.01$

Discussion

Phonatory function changes continuously from birth to old age due to fluctuations in the levels of sex hormones, differing significantly between sexes [3], with voice being a secondary sexual characteristic influenced by sex hormones [9]. A significant number of sex steroid receptors have been detected in human larynx tissues [3, 10, 11] suggesting a probable effect of these hormones on phonatory function and justifying their specific role in the development of the larynx [12].

During childhood the ovaries are not producing significant amounts of sex hormones as a result of hypothalamic and pituitary suppression. During these years, preceding puberty, the female larynx grows gradually and, with it the voice profile adjusts with no dramatic changes [13]. Comparisons of prepubertal, pubertal and adult female larynges revealed that the female larynx reaches adult size by puberty [14]. Vocal tract size also increases significantly at puberty [15], although the magnitude of the change is more prominent in boys than in girls. Laryngeal maturation caused by androgen stimulation after puberty is a fundamental basis for the difference between the male and female voice, although the direct mechanism for these are complex. Beckford et al. [16] investigated the effect of androgen stimulation to the laryngeal structures in a sheep model and reported that the thyroid cartilage demonstrated a greater response to male sex hormones than did the cricoid or arytenoid cartilage. Also, Claassen et al. [17] suggested that the involvement of androgen receptor-positive chondrocytes in thyroid cartilage mineralization, probably by a testosterone-linked stimulation of alkaline phosphatase. In addition, according to the Püschel and Nowakowsky's [18] study about the larynx in young castrated men, it should be taken into consideration that ossification of thyroid cartilage lags behind the normal ossification process in patients with androgen deficiency.

In the literature altered voice quality in women during menstrual cycle and menopause is assumed to be the result of hormonal changes [9, 19]. The changes in the female voice, frequently detected in clinical practice, are associated with the life cycle governed by sex hormones [19]. In addition, Claassen [20] reported that human thyroid cartilage mineralized sexual different, leading to whole ossification in males, but in females only the dorsal half of thyroid cartilage ossified while the ventral one remains unmineralized. And this had consequences for the gender-specific voice and, in addition for age-related changes of the voice. Also some studies have documented changes in the vocal quality of menopausal women, such as lack of intensity, vocal fatigue, low-voice and hoarse voice [6, 7], and reduction of fundamental frequency [6, 21] and have suggested probable beneficial effects of estrogen replacement therapy on the larynx for the prevention of changes in voice associated with menopause [22, 23].

Abitbol et al. [9] examined a group of 100 menopausal women using dynamic vocal exploration, stroboscopic examination, and cytologic smears. They reported that, in all women, vocal folds were less supple, had a thinner mucosa, and had reduced vibratory amplitude. Of these women, 17 were diagnosed with dysphonia associated with menopause. In this subgroup, they also noted unilateral or bilateral muscular atrophy of the vocal folds, asymmetric vibratory pattern, and discoloration of the mucosa with appearance of microvarices.

Some studies noted that professional singers sometimes complain of loss of brilliance, loss of power, and decreased ability to reach high notes after menopause [24]. Boulet and Oddens [25] used mailed questionnaires completed by 48 professional female singers. They reported that singers in their sixties often complained of vocal changes associated with menopause. Despite relatively large sample used, it should be noted that this study used only subjective self-reports. Thus, the possibility of a biased sample cannot be overruled.

In addition, Claassen et al. [26], around the time point of menopause, thyroid cartilages of men contained several

apoptotic chondrocytes while in women only a few or no apoptosis-positive chondrocytes were observed. In the age group 41–60 years, male specimens contained a significantly higher percentage of apoptotic chondrocytes than female ones. However, in the advanced age groups several apoptotic cells were seen in specimen of both genders. By comparing the percentage of apoptotic chondrocytes in thyroid cartilages of the age group 61–79 years no statistical significance was found between male and female specimens [26].

Recently studies have suggested that some alterations observed in the vocal folds of menopausal women such as edema, thickening and hyperemia may be due to the fall in estrogen levels and are attributed to the interstitial edema that sets in after menopause [7, 27]. According to some reports [28], the anatomical changes most commonly observed in menopausal women are edema and thickening of the vocal folds. However, definite information of histopathological changes of laryngeal mucosa due to estrogen deficiency, have not been studied, yet. Thus, we aimed to introduce the changes in larynx mucosa. Here, we observed significant changes 4 weeks after ovariectomy when we assessed subepithelial edema, inflammation, cilia and goblet cell loss.

Conclusion

Menopause is a critical event in women's lives. In the literature, estrogen deficiency causes symptoms and disorders in women especially in developed countries because of the increase in women's life expectancy has been reported [1, 6–8, 29]. Therefore, we thought that otolaryngological manifestations and data of histopathological changes due to estrogen deficiency will contribute to the literature. To date, the possible histopathological changes of larynx mucosa due to estrogen deficiency have not been studied. This study is the first paper describing the histopathological changes after estrogen deficiency in rats. Next step should be to evaluate the histopathological changes after hormone replacement therapy.

References

1. Amir O, Biron-Shental T (2004) The impact of hormonal fluctuations on female vocal folds. Curr Opin Otolaryngol Head Neck Surg 12(3):180–184
2. Mikkola T, Viinikka L, Ylikorkala O (1998) Estrogen and postmenopausal estrogen/progestin therapy: effect on endothelium-dependent prostacyclin, nitric oxide and endothelin-1 production. Eur J Obstet Gynecol Reprod Biol 79(1):75–82
3. Newman SR, Butler J, Hammond EH, Gray SD (2000) Preliminary report on hormone receptors in the human vocal fold. J Voice 14(1):72–81
4. Caruso S, Roccasalva L, Sapienza G, Zappalá M, Nuciforo G, Biondi S (2000) Laryngeal cytological aspects in women with surgically induced menopause who were treated with transdermal estrogen replacement therapy. Fertil Steril 74(6):1073–1079
5. Meurer EM, Wender MC, von Eye Corleta H, Capp E (2004) Phono-articulatory variations of women in reproductive age and postmenopausal. J Voice 18(3):369–374
6. Abitbol J, Abitbol B (1998) The voice and menopause: the twilight of the divas. Contracept Fertil Sex 26(9):649–655
7. Sataloff RT, Emerich K, Hoover CA (1997) Endocrine dysfunction. In: Sataloff RT (ed) Professional voice the science and art of clinical care. Singular Publish Group, California, pp 291–297
8. Raj A, Gupta B, Chowdhury A, Chadha S (2009) A study of voice changes in various phases of menstrual cycle and in postmenopausal women. J Voice 24(3):363–368
9. Abitbol J, Abitbol P, Abitbol B (1999) Sex hormones and the female voice. J Voice 13(3):424–446
10. Wu KH, Tobias ML, Kelley DB (2003) Estrogen receptor expression in laryngeal muscle in relation to estrogen-dependent increases in synapse strength. Neuroendocrinology 78(2):72–80
11. Claassen H, Mönig H, Sel S, Werner JA, Paulsen F (2006) Androgen receptors and gender-specific distribution of alkaline phosphatase in human thyroid cartilage. Histochem Cell Biol 126(3):381–388
12. Mendes-Laureano J, Sá MF, Ferriani RA, Reis RM, Aguiar-Ricz LN, Valera FC, Küpper DS, Romão GS (2006) Comparison of fundamental voice frequency between menopausal women and women at menacme. Maturitas 55(2):195–199
13. Wuyts FL, Heylen L, Mertens F, Du Caju M, Rooman R, Van de Heyning PH, De Bodt M (2003) Effects of age, sex, and disorder on voice range profile characteristics of 230 children. Ann Otol Rhinol Laryngol 112(6):540–548
14. Kahane JC (1982) Growth of the human prepubertal and pubertal larynx. J Speech Hear Res 25(3):446–455
15. Fitch WT, Giedd J (1999) Morphology and development of the human vocal tract: a study using magnetic resonance imaging. J Acoust Soc Am 106(3 Pt1):1511–1522
16. Beckford NS, Rood SR, Schaid D, Schanbacher B (1985) Androgen stimulation and laryngeal development. Ann Otol Rhinol Laryngol 94(6 Pt 1):634–640
17. Claassen H, Werner J (2004) Gender-specific distribution of glycosaminoglycans during cartilage mineralization of human thyroid cartilage. J Anat 205(5):371–380
18. Püschel L, Nowakowski H (1954) Ü ber den Einfluß der androgenen Hormone auf die Verknöcherung des Kehlkopfskelets. Arch Ohr-usw Heilk u Z Hals-usw Heilk 166:255–274
19. Sataloff RT (1987) Common diagnoses and treatments in professional singers. Ear Nose Throat J 66(7):278–288
20. Claassen Horst (1999) Untersuchungen zur Mineralisation und Knochenbildung im Schildknorpel und im ersten Rippenknorpel—Ein Beitrag zur Anatomie der sogenannten permanenten Knorpel. Shaker Verlag, Aachen
21. Stoicheff ML (1981) Speaking fundamental frequency characteristics of nonsmoking female adults. J Speech Hear Res 24(3):437–441
22. Lindholm P, Vilkman E, Raudaskoski T, Suvanto-Luukkonen E, Kauppila A (1997) The effect of postmenopause and postmenopausal HRT on measured voice values and vocal symptoms. Maturitas 28(1):47–53
23. Stemple JC, Glaze L, Klaben BG (2000) Pathologies of laryngeal mechanism. In: Stemple JC, Glaze L, Klaben BG (eds) Clinical

voice pathology: theory and management. Singular Thompson Learning, Canada, pp 85–143
24. Van Gelder L (1974) Psychosomatic aspects of endocrine disorders of the voice. J Commun Disord 7(3):257–262
25. Boulet MJ, Oddens BJ (1996) Female voice changes around and after the menopause—an initial investigation. Maturitas 23(1):15–21
26. Claassen H, Schicht M, Sel S, Werner J, Paulsen F (2009) The fate of chondrocytes during ageing of human thyroid cartilage. Histochem Cell Biol 131(5):605–614
27. Kiliç MA, Oğüt F, Dursun G, Okur E, Yildirim I, Midilli R (2004) The effects of vowels on voice perturbation measures. J Voice 18(3):318–324
28. Tonisi GABR (2000) Efeitos do climaterio na freqüencia fundamental. Rev CEFAC 2:73–80
29. Schneider B, Van Trotsenburg M, Hanke G, Bigenzahn W, Huber J (2004) Voice impairment and menopause. Menopause 11(2):151–158