Original Article

Effects of Ovariectomy on Rat Genioglossal Muscle Contractile Properties and Fiber-Type Distribution

Yue-hua Liu[a]; Shan-shan Jia[b]; Yu-xia Hou[b]

ABSTRACT

Objective: To test the hypothesis that ovariectomy has no effects on contractile, histochemical, or biochemical properties of the rat genioglossus (GG).

Materials and Methods: Eight-week-old female Sprague-Dawley rats were randomly assigned into three groups: normal group (Normal), sham-operated group (Sham), and ovariectomized group (OVX). Four weeks later, genioglossal electromyography activity (EMGgg) and contractile properties were measured, including relative integrated EMG (iEMG), maximal twitch tension, 70%-decay time, and fatigue index (FI). Then rats were sacrificed and paired GG were removed for further analysis. Adenosine-triphosphatase (ATPase) staining was performed to determine the percent fiber-type distribution and to identify cross-sectional area (CSA) of muscle fibers. Myosin heavy chain (MHC) phenotypes were determined by gel electrophoresis.

Results: Ovariectomy reduced EMG activity and contractile properties of the GG. Following ovariectomy, the CSA of type IIA and the proportion of MHCIIA decreased significantly. The MHC isoform composition of GG transferred from relative slow-twitch to fast-twitch isoform, following the order MHCIIB → MHCIIX → MHCIIA. Sham operation had no effect on any of the parameters.

Conclusions: The hypothesis is rejected. The contractile properties of the GG are sensitive to ovariectomy. These changes were, at least in part, associated with changes in the amount and type of contractile protein expressed. (*Angle Orthod.* 2009;79:509–514.)

KEY WORDS: Genioglossus; Ovariectomy; Electromyography; Myofiber phenotype; MHC

INTRODUCTION

Obstructive sleep apnea hypopnea syndrome (OSAHS) is a disorder that is characterized by repetitive sleep-induced collapse of the upper airway. The genioglossus (GG) is an important pharyngeal muscle that helps to maintain an open upper airway (UA) for effective breathing.[1] In patients with OSAHS, UA patency is compromised so that episodic apnea during sleep is due to collapse of the UA during inspiration.[2]

The prevalence of OSAHS has a strong male predominance with a male/female ratio between 2:1 and 4.9:1.[3] The prevalence of sleep apnea is higher in postmenopausal women without hormone replace treatment (HRT) than in premenopausal women. In contrast, postmenopausal women with HRT had a prevalence of OSAHS similar to that of premenopausal women. Popovic[4] reported that electromyographic activity of the genioglossus (EMGgg) was greater in the luteal phase as compared with the follicular phase of the menstrual cycle in premenopausal women. Furthermore, postmenopausal women demonstrated significantly less EMGgg compared with premenopausal women. It has been hypothesized that female hormones may have some impact on the contractile function of the GG, but the actual interaction between female hormones and UA patency is not yet known.

Skeletal muscle tissue is expected to be estrogen responsive because it has estrogen receptors with properties similar to those of classical target organs.[5,6] These receptors appear to be functional as suggested by scattered studies demonstrating diverse effects of estrogen on skeletal muscle. For example, estrogen seems to attenuate skeletal muscle damage as measured by the release of cytosolic enzymes or other markers.[7,8] Estrogen may increase the maximal activity

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of key lipid oxidation enzymes and may alter myosin heavy chain (MHC) isoform expression.[9,10] Finally, treating ovariectomized rats with estrogen reduces muscle mass and fiber size and alters maximal isometric contraction force.[11]

We hypothesized that female hormones may influence GG muscle function and fiber-type composition. The specific objective of the present study was to investigate the effects of ovariectomy on GG muscle structure and contractile properties.

MATERIALS AND METHODS

Experimental Animals and Surgical Procedures

Thirty 8-week-old female Sprague-Dawley (SD) rats (initial weight, 220 ± 20 g) were divided into three groups: (1) normal group (Normal, n = 10); (2) sham-operated group (Sham, n = 10); and (3) ovariectomized group (OVX, n = 10). Animals in the OVX group were anesthetized with pentobarbitone sodium (40 mg kg^{-1} I.P.), and bilateral ovaries were isolated and removed. For the Sham group, the abdominal wall was incised, but the ovaries were not removed. All animal protocols were approved by the Animal Care Committee of Tongji University.

Genioglossal electromyogram (EMGgg). Following the 4-week treatment period, animals were anesthetized with pentobarbitone sodium (40 mg kg^{-1} I.P.) and were given supplemental doses as needed to maintain deep anesthesia (no withdrawal to paw pinch). With rats supine, a ventral midline incision was made in the neck, and the genioglossus was isolated. Animals were tracheostomized and vagotomized bilaterally to maintain a patent airway. A bipolar, fine-wire EMG electrode (0.2 mm in diameter) with a separation of ≈3.0 mm between the poles was inserted (using a 28-gauge needle) into the muscle belly, parallel to the long axis of the muscle. The electrode was insulated except for the final 4 mm (length of the muscle belly), which terminated in a small hook in which muscle tissue could be anchored. Mineral oil was applied to keep the muscle moist during the experiment. Spontaneous EMG activity was recorded from each animal. Electrical signals were amplified (×400) and filtered (bandwidth of 10 to 300 Hz) with use of the SMUP-PC biomedical signal processing system (Fudan University, Medical College, Physiology Department, Shanghai, China). Raw signals were stored on a computer and were calculated to integrate the EMG (iEMG) with the use of MF lab functional experiment analysis software (Fudan University). The processed EMG signal was referred to as the relative integrated EMG ([iEMG in inspiration − iEMG in expiration]/iEMG in inspiration × 100%).

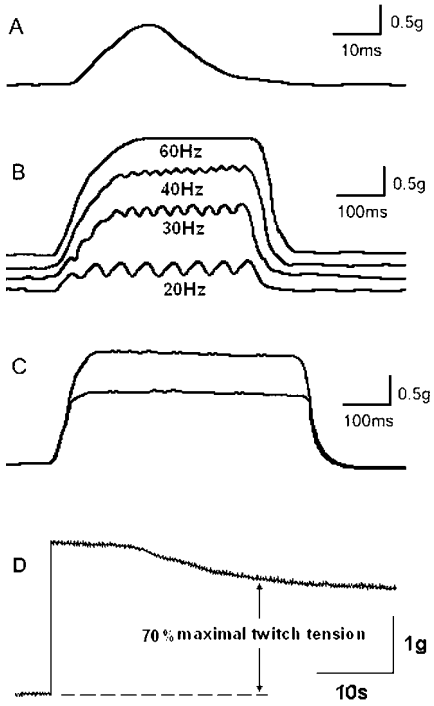

Figure 1. Contractile response of the rat genioglossus (GG). (A) Twitch characteristics in response to single pulse stimulation. Maximal single-twitch tension was elicited on stimulation at 1.86 V and 1 Hz. (B) Tetanic characteristics in response to stimulation at 20, 30, 40, and 60 Hz for 500 ms from bottom to top. GG fusion frequency = 60 Hz. (C) Fatigue response to stimulation at 60 Hz for 2 minutes. The first response is the top trace, and the last response is the bottom trace. FI = bottom tension/top tension. (D) Fatigue response to stimulation at 60 Hz. 70%-decay time is the duration of fusion frequency remaining at 70% of initial tension.

Contractile Properties

The tendon of the GG was cut at the mandible, and the other end of the GG was left intact. The dissociated tendon was attached to an isometric force transducer (ZL-2, Huajian, China) which was mounted to a vertical micropositioner. The optimal length (ie, the length that produced maximal isometric twitch tension) was determined, and the muscle was held at this length for the remainder of the experiment. Isometric twitch tension of the GG was measured in response to electrical field stimulation with platinum electrodes hooked onto the medial hypoglossal nerve. Maximal single-twitch tension was elicited by stimulating at 1.86 V, 1 Hz (Figure 1A). Then stimulation frequencies of 20, 30, 40, and 60 Hz were assessed at 1.86 V and 500 ms, and 60 Hz was chosen for use during the fatigue protocol because it approximated the fusion frequency (Figure 1B). The fatigue index (FI), which is the ratio of tension remaining after 2 minutes of 60 Hz stimulation to tension generated by the initial train, was used to express muscle fatigability (Figure 1C). The 70%-decay time is the duration of fusion frequency re-

maining at 70% of the initial tension (Figure 1D). The force transducer output was amplified and recorded on a computer via analog-to-digital conversion system (SMUP-PC). Data analysis was performed with the use of MF Labdat, version 3.0 (Fudan University).

Female Hormone Levels

Blood was collected by cardiac puncture and the uterus was harvested before animals were sacrificed. Blood samples were centrifuged and serum was stored at −80°C prior to analysis. Estradiol and progestogen levels were analyzed by the radioimmunity method (Progesterone Radioimmunoassay kit and Estradiol Radioimmunoassay kit, BeiFang Biotechnology Company, Beijing, China).

Myosin ATPase Staining

After all animals were sacrificed, paired GG were quickly removed. One was immediately frozen in liquid nitrogen and stored at −80°C for adenosine triphosphatase (ATPase) staining; the other part also was stored at −80°C for major histocompatibility complex (MHC) analysis. Ten-μm sections were cut from muscle samples with a cryostat at −22°C and were dried at room temperature. Two neighboring sections were stained for acid and alkali-labile ATPase activity.[12] Slices were subjected to a modified procession: one slice was incubated for 10 minutes in 0.1 M sodium acetate solution (pH 4.6, 4°C). After they were rinsed with distilled water, slices were incubated together in an ATPase substrate solution for 30 minutes (pH 9.6, 37°C). After undergoing rinsing, slices were incubated in 2% CoCl solution for 5 minutes, rinsed with distilled water thoroughly, and dipped in 10% ammonium sulfide (1 to 2 minutes). Muscle fibers were classified according to conventional criteria. Morphometric analysis was performed with an Olympus microscope at ×20 magnification, with connection to a computer. Boundaries of individual muscle fibers were delineated, and fiber cross-sectional area (CSA) was determined from the number of pixels within the outlined fiber (Figure 2). At least 50 fibers of each sample were used to calculate mean CSA of all fiber types.

Electrophoretic Determination of MHC

The MHC composition of the GG was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Frozen muscles were minced with scissors in 9 vol of ice-cold homogenization buffer (250 mM sucrose, 100 mM KCl, 5 mM EDTA, 20 mM Tris, pH 6.8).[13] The muscle minces were subsequently homogenized in glass tissue grinders. After they were stirred for 30 minutes on ice, the homogenates were

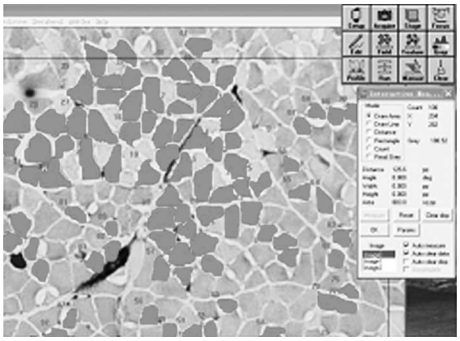

Figure 2. Cross-sectional area (CSA) was determined from the number of pixels within the outlined fiber.

centrifuged at 10,000 × *g* for 15 minutes (4°C). Supernatants were separated and stored at −20°C. Total protein was assayed according to the method of Bradford.[12] Before electrophoresis, supernatants were diluted 1:1 (v/v) with 2× Laemmli buffer and were boiled for 3 minutes. For each sample, 40 μg of protein was loaded per lane. MHC composition was analyzed on 10% modified separation gels (Arc:Bis 30:0.3, 10% glycerol, 2% SDS, 595 mM Tris-HCl, pH 8.8) and 4% stacking gels (Arc:Bis 30:1, 2% SDS, 25 mM Tris-HCl, pH 6.8). Electrophoresis was carried on for 6 hours at constant voltage (70 v) and at 4°C. Gels were stained with Coomasie Blue. The relative amount of each MHC isoform was determined by densitometry.

Data Analysis

Means and standard deviations were calculated for all dependent variables. Data were analyzed by one-factor analysis of variance (ANOVA) to test for differences among the treatment groups. The least significant difference (LSD) post-hoc analysis was used to determine which groups were significantly different from one another when the ANOVA suggested a significant group effect. Statistical significance was accepted at the .05 level.

RESULTS

Female Hormone Levels and Uterine Weights

Compared with the Normal group, estrogen and progesterone levels decreased significantly in the OVX group ($P < .05$, $P < .01$). Evidence of ovariectomy also was indicated by the 70% reduction in uterine weight in the OVX group ($P < .01$) (Table 1). No significant difference was detected between Normal and Sham groups.

Genioglossal Electromyogram (EMGgg)

The EMGgg was characterized as phasic discharge in inspiration, and the discharge was substantially re-

Table 1. Levels of Serum Estrogen and Progesterone in Three Groups[a]

	Normal (n = 10)	Sham (n = 10)	OVX (n = 10)
Estrogen, ng/L	321.74 ± 33.07	334.50 ± 38.15	237.21 ± 29.34*
Progesterone, μg/L	1.70 ± 0.07	1.68 ± 0.06	0.75 ± 0.07**
Uterine weights, mg	550 ± 53.48	552.5 ± 58.52	158.0 ± 8.37**

[a] Values are listed as means ± standard deviations. Statistical comparison was made against the Normal group.

* $P < .05$; ** $P < .01$.

duced (although not eliminated) during expiration. Relative iEMG in inspiration was compared among groups. iEMG decreased significantly in the OVX group when compared with the Normal group ($P < .05$) (Figure 3, Table 2). No significant difference was detected between Normal and Sham groups.

Contractile Properties

Contractile properties of the rat GG are summarized in Table 2. Maximal twitch tension, 70%-decay time, and FI decreased significantly in the OVX group as compared with the Normal group. No significant difference was detected between Normal and Sham groups.

Myosin ATPase Staining

Composition and mean CSA of type IIA fibers are summarized in Figure 4 and Table 3. No significant difference in relative ratio was detected among the three groups ($P > .05$), but the CSA of type IIA fiber decreased significantly in the OVX group compared with the Normal group ($P < .001$).

Electrophoretic Determination of MHC

MHC isoforms of rat GG were predominantly composed of MHCIIA and MHCIIB. (Composition is summarized in Figure 5; Table 4.) No significant difference was recognized between Normal and Sham groups. The proportion of MHCIIA decreased significantly in the OVX group ($P < .01$), and the relative content of MHCIIX and MHCIIB increased compositely ($P < .01$).

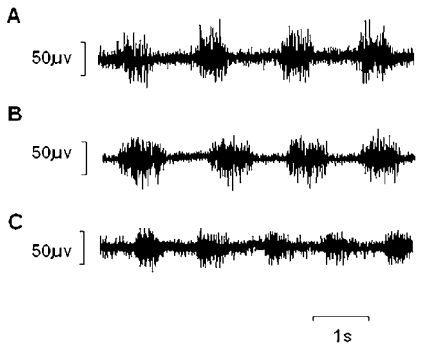

Figure 3. Genioglossal electromyography activity (EMGgg) was decreased significantly after ovariectomy. (A) Normal group. (B) Sham group. (C) Ovariectomized group (OVX).

Table 2. Electromyography and Contractile Properties of Rat Genioglossus in Three Groups[a,b]

	Normal (n = 10)	Sham (n = 10)	OVX (n = 10)
Maximal twitch tension, g	3.95 ± 0.49	3.85 ± 0.52	3.22 ± 0.30*
70%-decay time, ms	26.0 ± 2.83	25.5 ± 3.12	20.0 ± 3.07*
Fatigue index	0.62 ± 0.04	0.59 ± 0.03	0.42 ± 0.03**
Relative iEMG, %	95.76 ± 20.36	88.99 ± 19.03	60.91 ± 13.17*

[a] Values are listed as means ± standard deviations. Statistical comparison was made against the Normal group.

[b] iEMG indicates integrated electromyography; OVX, ovariectomized.

* $P < .05$; ** $P < .01$.

DISCUSSION

In the present study, we examined the effects of ovariectomy on contractile, histochemical, and biochemical properties of rat GG. The GG exhibits a respiration-related activity pattern. EMGgg activity increased during inspiration, and the discharge was substantially reduced during expiration. In this study, EMGgg decreased significantly following ovariectomy, which suggested that female hormones may have a substantial impact on GG activity. One of our key findings was that ovariectomy reduced GG muscle fatigue resistance. Related studies reported that maximal isometric tetanic forces of EDL and soleus were significantly decreased in OVX mice; these were increased

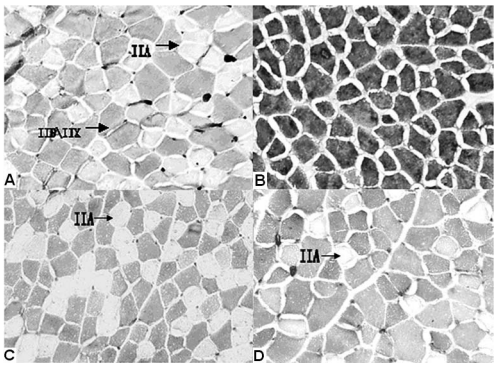

Figure 4. Adenosine triphosphatase (ATPase) staining for fiber phenotype of rat genioglossus (GG). (A) Normal group. Type IIA (light staining) and IIB/X (moderately dark staining) fibers in acidic media (pH, 4.6). (B) Normal group. Type II (dark staining) fibers in alkaline (pH, 9.6) media. No type I (light staining) fibers found. (C and D) Cross-sectional area (CSA) of type IIA fibers decreased in the ovariectomized (OVX) group compared with the Normal group.

Table 3. Myofiber Composition and CSA of GG in Three Groups[a,b]

	Normal (n = 10)	Sham (n = 10)	OVX (n = 10)
Type IIA, %	40.37 ± 1.01	39.80 ± 1.02	39.40 ± 1.22
Type IIB/IIX, %	59.73 ± 1.01	60.20 ± 1.02	60.60 ± 1.22
Type IIA, μm^2	1198.00 ± 72.47	1172.60 ± 80.46	898.70 ± 133.01**
Type IIB/X, μm^2	1632.00 ± 140.29	1625.20 ± 149.97	1677.60 ± 144.67

[a] Values are listed as mean ± standard deviations. Statistical comparison was made against the Normal group.
[b] CSA indicates cross-sectional analysis; GG, genioglossus.
* $P < .05$; ** $P < .01$.

by about 14% after estrogen replacement. These findings are consistent with those of previous studies.

A limitation of this study is that the ATPase staining procedure did not permit us to distinguish between type IIB and IIX fibers, but MHC protein isoforms were separated with use of the SDS-PAGE protocol. The myofiber of the GG was composed exclusively of type II fibers (IIA, 40.37 ± 1.01%; IIB/IIX, 59.73 ± 1.01%). Only a fewer scattered type I fibers were found. This result was similar to that reported in Volz et al's[14] study. The effects of sex hormones on skeletal muscle structure and function are controversial. Ovariectomy was reported to have no effect on soleus muscle structure in rats.[15] In another study, it was reported that ovariectomy reduced fast MHC expression in the rat soleus muscle[10]; however, in our study, the CSA of type IIA fibers and the expression of MHCIIA isoform both were decreased significantly in the OVX group. A shift from slow to fast isoform also was observed in the GG following ovariectomy. Because MHC isoform changes tend to follow a general pathway of sequential transition in the order MHC I ↔ IIA ↔ IIX ↔ IIB,[16] our findings might be interpreted as (1) an overall transition in the expression of fast isoforms toward slower isoforms, or (2) specific upregulation of the slower isoforms of MHC genes, or (3) specific downregulation of genes coding for fast MHC isoforms following ovariectomy. Translation of the MHC isoform from MHCIIA to MHCIIB may induce GG to be more vulnerable to fatigue. Rivero[17] reported that the greatest average levels of succinate dehydrogenase (SDH) activity occur in MHCIIA fiber, which produces ATP through aerobic oxidation in the mitochondria, where 18 ATP molecules are produced from one acetate residue, with only carbon dioxide and water as by-products. In contrast, the highest level of glycerophosphate dehydrogenase (GPD) activity occurs in type MHCIIB fiber, which produced ATP through anaerobic glycolysis. This anaerobic glycolysis generates one ATP and two lactate or pyruvate molecules from one glucose molecule, and the by-products (lactate molecules) lead to cytosol acidification, which inhibits muscle contractile response and causes fatigue. Furthermore, the glycogen store is depleted more rapidly when large amounts of lactic acid are produced anaerobically. Finally, muscle performance will be severely depressed at low glycogen levels.[18,19]

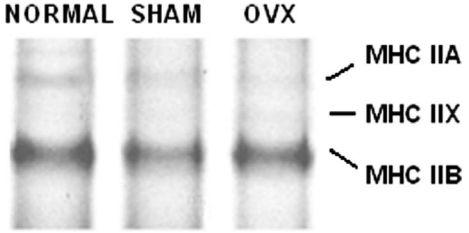

Figure 5. Major histocompatibility complex (MHC) isoform composites of rat genioglossus (GG). A representative gel is shown. A control diaphragm sample was included on each gel to show resolution of type IIA, IIB, and IIX MHC isoforms.

Abnormalities in a variety of UA muscles have been observed in OSAHS patients.[20,21] Abnormalities of the sternohyoid and the geniohyoid have been observed in the English bulldog, an animal model of human OSAHS.[22] In all of these studies, an increase in fast-twitch fibers was reported; this has been suggested to be due to chronically increased activity and force generated in the UA muscles.[23] If our results from animals were to hold true for humans, the higher prevalence of OSAHS in postmenopausal women would be understandable, and HRT may be helpful for patients' UA muscles. Further work is needed to investigate the effects of exogenous sex hormone and the mechanism of action on GG.

CONCLUSIONS

- Results reported here demonstrate that EMG activity and contractile properties of the GG are sensitive to

Table 4. MHC Isoforms: Composition of GG in Three Groups[a,b]

	Normal (n = 10)	Sham (n = 10)	OVX (n = 10)
MHCIIA, %	28.54 ± 1.15	26.80 ± 0.99	9.42 ± 1.03**
MHCIIX, %	6.48 ± 0.47	6.08 ± 0.22	9.74 ± 0.51**
MHCIIB, %	64.66 ± 2.78	64.50 ± 3.99	80.86 ± 6.58**

[a] Values are listed as mean ± standard deviations. Statistical comparison was made against the Normal group.
[b] GG indicates genioglossus; MHC, major histocompatibility complex.
* $P < .05$; ** $P < .01$.

ovariectomy. These changes were, at least in part, associated with changes in the amount and type of contractile protein expressed.
- These results show that deficiency of female hormones in rats decreases MHCIIA isoform distribution and reduces type IIA fiber CSA.
- These effects may contribute to the protective effects of estrogen on the patency of the upper airway and the pathogenesis of OSAHS.

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REFERENCES

1. Remmers JE, De Groot WJ, Sauerland EK, Anch AM. Pathogenesis of upper airway occlusion during sleep. *J Appl Physiol: Respirat Environ Exercise Physiol.* 1978;44:931–938.
2. Deegan PC, McNichoals WT. Pathophysiology of obstructive sleep apnoea. *Eur Respair J.* 1995;8:1161–1178.
3. Quintana-Gallego E, Carmona-Bernal C, Capote F, Schez-Armengol A, Botebol-Benhamou G, Polo-Padillo J, Castillo-Gez J. Gender differences in obstructive sleep apnea syndrome: a clinical study of 1166 patients. *Respir Med.* 2004; 98:984–989.
4. Popovic RM, White DP. Upper airway muscle activity in normal women: influence of hormonal status. *J Appl Physiol.* 1998;84:1055–1062.
5. Dahlberg E. Characterization of the cytosolic estrogen receptor in rat skeletal muscle. *Biochim Biophys Acta.* 1982; 717:65–75.
6. Gruber B, Cohen L, Blix PM. Specific cytosol binding of diethylstilbestrol in rat skeletal muscle. *Steroids.* 1982;39: 479–495.
7. Stupka N, Lowther S, Chorneyko K, Bourgeois JM, Hogben C, Tarnopolsky MA. Gender differences in muscle inflammation after eccentric exercise. *J Appl Physiol.* 2000;89: 2325–2332.
8. Tiidus PM. Influence of estrogen on skeletal muscle damage, inflammation, and repair. *Exerc Sport Sci Rev.* 2003; 31:40–44.
9. Campbell SE, Febbraio MA. Effect of ovarian hormones on mitochondrial enzyme activity in the fat oxidation pathway of skeletal muscle. *Am J Physiol.* 2001;281:E803–E808.
10. Kadi F, Karlsson C, Larsson B, Eriksson J, Larval M, Billig H, Jonsdottir IH. The effects of physical activity and estrogen treatment on rat fast and slow skeletal muscles following ovariectomy. *J Muscle Res Cell.* 2002;23:335–339.
11. Warren GL, Lowe DA, Inman CL, Orr OM, Hogan HA, Bloomfield SA, Armstrong RB. Estradiol effect on anterior crural muscle-tibial bone relationship and susceptibility to injury. *J Appl Physiol.* 1996;80:1660–1665.
12. Jaffe DM, Terry RD, Spiro AJ. Disuse atrophy of skeletal muscle: a morphometric study using image analysis. *J Neurol Sci.* 1978;35:189–200.
13. Talmadge RJ, Roy RR. Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. *J Appl Physiol.* 1993;75:2337–2340.
14. Volz LM, Mann LB, Russell JA, Jackson MA, Leverson GE. Connor NP. Biochemistry of anterior, medial and posterior genioglossus muscle in the rat. *Dysphagia.* 2007;22:210–214.
15. McCormick KM, Burns KL, Piccone CM, Gosselin LE, Brazeau GA. Effects of ovariectomy and estrogen on skeletal muscle function in growing rats. *J Muscle Res Cell Motil.* 2004;25:21–27.
16. Pette D, Staron RS. Transitions of muscle fibre phenotypic profiles. *Histochem Cell Biol.* 2001;115:359–372.
17. Rivero JLL, Talmadge RJ, Edgerton VR. Fiber size and metabolic properties of myosin heavy chain–based fibre types in rat skeletal muscle. *J Muscle Res Cell Motil.* 1998;19: 733–742.
18. van der Laarse WJ, Lannergren J, Diegenbach PC. Resistance to fatigue of single muscle fibres from Xenopus related to succinate dehydrogenase and myofibrillar ATPase activities. *Exp Physiol.* 1991;76:589–596.
19. Polla B, D'Antona G, Bottinelli R. Respiratory muscle fibres: specialization and plasticity. *Thorax.* 2004;59:808–817.
20. Series F, Cote C, Simoneau J-A, Gelinas Y, St Pierre S, Leclerc J, Ferland R, Marc I. Physiologic, metabolic, and muscle fiber type characteristics of musculus uvulae in sleep apnea hypopnea syndrome and in snorers. *J Clin Invest.* 1995;95:20–25.
21. Series F, Simoneau J-A, St Pierre S, Marc I. Characteristics of the genioglossus and musculus uvulae in sleep apnea hypopnea syndrome and in snorers. *Am J Respir Crit Care Med.* 1996;153:1870–1874.
22. Petrof BJ, Pack AI, Kelly AM, Eby J, Hendricks JC. Pharyngeal myopathy of loaded upper airway in dogs with sleep apnoea. *J Appl Physiol.* 1994;76:1746–1752.
23. Petroff BJ, Hendricks JC, Pack AI. Does upper airway muscle injury trigger a vicious cycle in obstructive sleep apnea? A hypothesis. *Sleep.* 1996;19:465–471.