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Impaired contractility of the circular striated urethral sphincter muscle may contribute to stress urinary incontinence in femal zucker fatty rats

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Abstract

AIM—Obesity has been an independent risk factor for female stress urinary incontinence (SUI), the mechanism of this association remains unknown. The aim of this study is to validate the hypothesis that urethral dysfunction is a possible contributor to SUI in obese women.

METHODS—Ten Zucker Fatty (ZF) (ZUC-Lepr^{fa} 185) and 10 Zucker Lean (ZL) (ZUC-Lepr^{fa} 186) female rats at 12-week-old were used in this experiment. The urethral sphincter rings were harvested from the bladder neck through to the most proximal 2/3 regions. In the organ bath study, single pulses of electrical field stimulation (EFS) were applied. For the fatiguing stimulation, repeated multi-pulse EFS with 70 mA were applied at frequency of 5 Hz for 5 min. Caffeine-containing Krebs' solution was administrated to contract the urethra until the contraction began to reach a plateau for 10 min. We performed immunofluorescence staining of the urethra after the experiment was finished.

RESULTS—Compared to ZL controls, ZF rats had significantly impaired muscle contractile activity (MCA) ($P < 0.05$). Also, ZF rats presented early fatiguing of MCA and had a significantly greater percentage of MCA decline from baseline in the fatiguing test (37.7% vs 25.6%, $P < 0.05$). The plateau of maximal MCA induced by caffeine in ZF rats was significantly lower than ZL controls (0.22 vs 0.36, $P < 0.05$).

CONCLUSIONS—This novel study showed that obese female rats had significantly impaired contractile properties of striated urethral sphincter, suggesting urethral dysfunction could be an important contributor to SUI in obesity.

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POTENTIAL CONFLICTS OF INTEREST

Nothing to disclose.

Keywords

electrical field stimulation; female rats; obesity; organ bath; urethral sphincter

1 | INTRODUCTION

Obesity is a modern epidemic. It has become a main public health concern in the 21st century due to the rapidly increasing prevalence in recent years. The worldwide prevalence of obesity has doubled from 1980 to 2008.[1] In a recent analysis of data from the 2009 to 2010 National Health and Nutrition Examination Survey, Ogden et al[2] found that up to 35.7% of American adults were obese, which remarkably increased the occurrence of associated high-risk comorbidities and placed great financial burden on healthcare services.

In fact, obesity presents not only with well-described major medical conditions but also a number of quality-of-life issues. In women, there has been growing evidence about the relation between obesity and the prevalence of urinary incontinence.[3] Chen et al[4] used the Sandvik incontinence severity scale to show that obesity is a significant risk factor for urinary incontinence (OR = 4.1) and that obese women reported a higher urinary incontinence severity score, reaching statistical significance. Many of the epidemiological studies further revealed a clear dose-response effect of weight on urinary incontinence with each 5-unit increment in BMI associated with about a 20–70% increase in the risk of urinary incontinence, which is most strongly associated with stress urinary incontinence (SUI).[5]

Although compelling literature have documented that obesity is a significant independent risk factor for female SUI, the mechanism of this association is still unknown. Historically, the hypermobility theory, which considers that female SUI is predominantly associated with a lack of urethral support, has dominated the field.[6] However, it is well known that many women with urethral hypermobility remain continent, and surgical improvement of urethral support still fails a significant number of women.[7] Furthermore, a recent case-control study by DeLancey et al[8] demonstrated that changes in the urethra play the most significant role in female SUI development. With this information in mind, there is a clear need to refocus our attention on the urethra itself.

The urethra appears to be a critical but yet relatively understudied structure that likely contributes to the increased female SUI observed with obesity. However, there are no published studies that directly address the impact of obesity on urethral function mechanisms. To validate the hypothesis that urethral dysfunction is a contributor to SUI in obese women, we investigated the contractile properties of the urethral striated sphincter by comparison of obese and lean female rats in an ex vivo organ bath study. This novel study provides the opportunity to further understand the alterations of female urethral function associated with obesity that may result in female SUI.

2 | MATERIALS AND METHODS

2.1 | Animals

Ten Zucker Fatty (ZUC-FA/FA) (ZUC-Lepr^{fa} 185) (ZF) and 10 Zucker Lean (ZUC-LEAN) (ZUC-Lepr^{fa} 186) (ZL) female rats at 12-week-old were used in this experiment. All rats were obtained from Charles River Laboratories (Wilmington, MA). Urine leakage from the external urethral orifice can be observed when light pressure was applied to the suprapubic area in ZF rats. The animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at our institution.

2.2 | Ex vivo organ bath study

The urethral sphincter was harvested from both ZF and control ZLrats and placed inorganbath to measuremuscle contractility as previously described in 2010.[9] In brief, rats were anesthetized with intraperitoneal injection of pentobarbital (200 mg/kg). After a midline abdominal incision was made, the abdomen was opened and the pubic symphysis divided to expose the urinary bladder and the underlying urethra. The bladder was isolated together with the proximal 2/3 of the urethra, which was cut transversely. The proximal 2/3 of urethra was removed as a single ring from each rat then mounted transversely in a vertical tissue bath (Myobath Tissue Bath System II, World Precision Instruments, Sarasota, FL). Each 10 mL-chamber of which was filled with Krebs solution (NaCl, 86.87 mM; KCl, 5.16 mM; $MgSO_4$, 1.22 mM; $NaHCO_3$, 25.56 mM; $CaCl_2$, 1.33 mM, and dextrose, 1.01 mM, pH 7.6) that was maintained at 37°C and continuously aerated with 95% O_2 and 5% CO_2. Through an L-shaped metal wire, the ring-shaped urethral segment was connected to a force-displacement transducer (FORT 25, World Precision Instruments).

Before the experiment started, we performed equilibration of the force transducer to ensure the comparability of dataset generated at different channels and at different time. In brief, the baseline voltage number (V_0) for zero gram was recorded firstly with the data acquisition system (Lab TRAX-4, World Precision Instrument), 5 gm standard weight was then applied to force transducer and the voltage number (V_5) was recorded. A k number for converting voltage to force in g was calculated with equation followed: $k = (V_5 - V_0)/5$. In each experiment, the recorded voltage (V_s) was then converted into force in gram by equation followed: $F(g) = V_s/k$. Therefore, a master equation was applied in our experiment to convert the voltage into force in gram: $F(g) = V_s \times 5/(V_5 - V_0)$. Before experiment, the length and weight of entire isolated proximal 2/3-urethra tube were measured post removal and the urethra was stretched out to original length. The muscle contractile activity (MCA) was expressed in Newtons per square centimeter (N/cm^2) and then the MCA was calculated using the following formula: MCA (N/cm^2) = (force (g) × muscle length (cm) × 1.06)/(muscle weight (g) × 0.00981).[10]

2.3 | Electrical field stimulation (EFS)

To investigate the muscle contractile response to electrical stimulation, EFS was applied through a pair of platinum electrodes placed on either side of each preparation. The electrodes run the length of the preparation and were positioned a sufficient distance (~0.5 cm) apart to ensure that the muscles are field stimulated and not by direct stimulation

(contact) with an electrode. Then EFS was applied using an electronic stimulator connected to the electrodes. A constant current device was used to keep the current stable. For EFS with single pulses, square-wave pulses of 10 ~ 70 mA intensity and 0.2 ms in duration were applied with a 3-min interval between each activated contraction. For the fatiguing stimulation, repeated multi-pulse EFS with square-wave pulses of maximal intensity (70 mA) were applied at frequency of 5 Hz for 5 min. Both of single pulses and fatiguing stimulations were performed for each of the 10 ZF and 10 ZL rats.

2.4 | Contractile responses induced by Caffeine

We investigated the Caffeine induced muscle contractile response following the EFS experiments for each rat. Caffeine, as a releaser of calcium from intracellular stores, has been used to stimulate striated muscle contraction in several animal studies.[11] Caffeine was obtained from Sigma-Aldrich (St. Louis, MO). Caffeine was initially dissolved in citrate buffer and then dissolved in Krebs' solution to the concentration of 40 mM. When the experiment started, the Krebs' solution in an organ bath was exchanged by adding caffeine-containing Krebs' solution to contract the urethra until the contraction of each specimen began to reach a plateau for 10 min. The isometric tension was recorded with the data acquisition system (Lab TRAX-4, World Precision Instrument).

2.5 | Immunofluorescence staining

We performed H&E and immunofluorescence staining of the urethra for each rat after the experiment was finished. Tissue was fixed in cold 2% formaldehyde and 0.002% saturated picric acid in 0.1 MPBS, pH 8.0, for 4 h followed by overnight immersion in 0.1 MPBS containing 30% sucrose. The specimens were then embedded in OCT compound (Sakura Finetic USA, Torrance, CA). Frozen sections were incubated overnight with primary antibodies including anti-myosin skeletal heavy chain (MHC; 1:500; Mouse, [MY-32] [ab51263], [Q2]Abcam) and anti-smooth muscle actin (SMA; 1:1000; Mouse, Abcam). Secondary antibodies used included Alexa-488- and Alexa-594-conjugated antibodies (1:500; Invitrogen). Nuclei were stained with DAPI.

2.6 | Statistical analysis

Data were presented as means ± standard deviation (SD). Isometric contractile responses were given as g contraction/mm^3 tissue volume. Statistical significance between two groups was analyzed by the Student *t*-test. For statistical significance among multiple groups, one- or two-way ANOVA analysis followed by Bonferroni post hoc analysis was performed using the SAS software (SAS Institute Inc., Cary, NC).

3 | RESULTS

3.1 | Immunofluorescence staining of the urethra used in experiment

To identify the striated muscle fibers in the isolated female rat urethra, we performed immunofluorescence staining of the urethra for each rat after the experiment was finished. Figure 1 clearly shows that the striated muscle fibers (MHC staining) were included in the isolated urethra used in this study.

3.2 | Defect of urethral striated MCA induced by single-pulse EFS in obese rats

The single-pulse EFS successfully induced urethral striated MCA ex vivo (Fig. 2). Ten rats from both groups were used. The contraction responses stimulated by different single-pulse EFS intensity were recorded for each rat. There were stepwise increases of MCA associated with an incremental increase of individual stimulations from 30 to 70 mA in both groups. However, compared to ZL controls, ZF rats had significantly impaired MCA (N/cm^2, mean ± SD) (170.0 ± 112.2 vs 368.0 ± 280.3 at 30 mA and 575.5 ± 366.3 vs 910.8 ± 444.8 at 70 mA, respectively, $P < 0.05$). Also, the ZF rats had lower percentage of MCA increment from baseline (30 mA) than ZL controls (867% vs 1083% increment at 70 mA, $P < 0.05$).

3.3 | Fatiguing stimulation test induced by repeated multipulse EFS

In the investigations of the urethral striated muscle fatiguing stimulation, the fatiguing decline profile of the urethral striated MCA in both groups was tested (Fig. 3). Compared to ZL controls, ZF rats had early fatiguing of MCA and also had a significantly greater percentage of MCA decline from baseline in a 5-min fatiguing test (37.7 ± 10.5% vs 25.6 ± 11.8%, $P < 0.05$).

3.4 | Urethral striated MCA stimulated by Caffeine

Figure 4 shows the MCA profile stimulated by caffeine in both groups. Compared to ZL controls, the MCA induced by caffeine in ZF rats was earlier to reach a plateau. The plateau of maximal MCA (N/cm^2, mean ± SD) in ZF rats was significantly lower than in ZL controls (0.22 ± 0.15 vs 0.36 ± 0.15, $P < 0.05$).

4 | DISCUSSION

Obesity is a major health problem all over the world. In addition to increasing the risk of metabolic and cardiovascular disease, obesity has strongly been linked to urinary incontinence. Published studies have shown that the rate of urinary incontinence in females is increasing secondary to concomitant rising rates of obesity.[12] Current research reveals that obese women on a weight loss program show a significant urinary incontinence improvement, of which a 5% weight loss led to a 50% reduction in urinary incontinence episodes.[13] In longitudinal cohort studies, obesity was also found to be associated with prevalent and new onset urinary incontinence during 5–10 years of follow up. The odds of incident urinary incontinence increased by approximately 7–12% for each 1 kg/m^2 unit increase in BMI.[14]

Although most of the epidemiological studies have identified obesity to be an established risk factor for causing female urinary incontinence and SUI is the predominant form of incontinence studied, little is known about the mechanism for this association. In 2008, Hunskaar[15] proposed that obesity causes female SUI because the added body weight, like pregnancy, results in increased abdominal pressure, which in turn leads to weakening of the pelvic floor innervation and musculature. It would then logically follow that increased weight and abdominal pressure would result in increased bladder pressure and increased urethral mobility. However, the scientific basis for these proposed mechanisms remains relatively weak. Although loss of anatomic support due to obesity may play an important

role in the development of female SUI, other changes seen also contribute to the development of an incompetent urethra. In 2010, Gasbarro et al[16] studied the effects of obesity on the lower urinary tract by evaluating voiding function and urethral anatomy in female rats. Histological study of the striated urethral sphincter in obese rats revealed increased fibrosis and edema, leading to marked disruption of the striated muscular structure compared with controls. However, they used a different animal model, ZDF (Zucker Diabetic Fatty) rat, while we used ZF in our experiment. They are different from each other in terms of genotype and phenotype. ZDF rats developed progressive insulin resistance and glucose intolerance between 3 and 8 weeks of age and become overtly diabetic between 8 and 10 weeks of age.[17] While the ZF rat is considered as a model for pre-diabetes, as characterized by a genetic defect that results in obesity and other characteristics of metabolic syndrome. These animals become glucose intolerant but do not develop type 2 diabetes.[18]

Within the female urethra, the striated sphincter has been suggested as a possible mechanism for maintenance of continence.[19] The urethral striated muscle of obese women with SUI is significantly reduced, and this decline parallels the decrease in maximal urethral closure pressure.[20] As such, dysfunction or irreversible damage to the striated urinary sphincter in obesity can potentially result in the development of SUI. In 2015, Lee et al[21] reported that obesity would accelerate the onset of striated muscle wasting by decreasing the myofiber area, satellite cells, and myonuclei of the gastrocnemius muscle. However, the authors did not examine the pelvic floor or the urethral striated muscles. To better define the urethral mechanism involved in the pathophysiology of female SUI in obesity, measurement of striated sphincter contractile function appears to be an important endpoint.

This is the first study to investigate the impact of obesity on contractile properties of urethral striated sphincter in female rats. The assessment of isolated urethral function ex vivo offers the advantage of investigating contractile properties of striated sphincter in the absence of any influences from the nerve or blood supply. Our major findings are: (i) compared to ZL controls, ZF rats had significantly impaired contractile responses evoked by EFS with single pulses and application of caffeine; and (ii) in the fatiguing test stimulated by repeated multi-pulse EFS, the striated urethra of ZF rats presented early fatiguing and had significantly greater percentage of contractility decline from baseline. These findings provide direct evidence of urethral dysfunction in female rats of obesity, suggesting urethral incompetence could be an important contributor to SUI in obese women.

In women, the circular striated muscle of the urethra is a critical contractile element of the sphincteric mechanism, which must generate sufficient resistance when bladder pressures increases suddenly, to prevent SUI. While it is well known that the female rat urethral musculature comprises an inner longitudinal smooth muscle layer, a middle circular smooth muscle layer, and an outer striated muscle layer, the distribution of these layers along the length of the urethra remains disagreeable even between dedicated studies. Specifically, while Kim et al[22] reports highly variable distribution of the striated muscle along the length of the urethra, a recent study by Lim et al[23] reports a nearly uniform distribution of the striated muscle along the entire length of the urethra. Very recently, in rats, we have confirmed that the striated muscle is confined to the proximal 2/3 of the urethra similar to the structure in women.[24] The organ bath assay in current study was carried out on the

urethra ring that was mounted transversely, and therefore only the circular striated muscle activity was measured. The rings were taken from the bladder neck through to the most proximal 2/3 region of the urethra, enabling experimentation along the entire functional length of the striated sphincter. Although impaired contractile properties of urethral striated sphincter were demonstrated in obese rats, the exact mechanisms linking these two conditions are not clear. However, the associations between obesity and increased abdominal pressure were reported in recent studies.[25] The increased abdominal pressure could cause chronic strain, stretching, and weakening of the urethral muscles, and thus increase the risk of SUI.[26] On the other hand, several molecular investigations have shown that obesity is associated with increased adipocytokine production and proinflammatory activity, leading to disturbances in oxidative metabolism and insulin resistance, which in turn may damage the vascular system in the pelvic floor and result in a dysfunction of urethral sphincter muscles.[27] Furthermore, recent studies have demonstrated that striated muscle regeneration occurs in two major steps—the activation of muscle stem cells and satellite cells, and their differentiation.[28] Obesity can cause striated muscle dysfunction by reducing both steps as well as increasing the expression of the myogenesis-inhibiting factor myostatin.[29] These adverse effects induced by obesity may potentially contribute to urethral striated sphincter dysfunction, leading to the development of female SUI.

In summary, the present study showed that obese female rats had significantly impaired contractile properties of the urethral striated sphincter, suggesting urethral dysfunction could be an important contributor to SUI in obese women. These findings enable us to determine the contribution of obesity to urethral dysfunction in female rats and to give new insight into the characterization of the urethral mechanism involved in female SUI in obesity. However, the ZF rat is considered as a model for pre-diabetes, as characterized by a genetic defect in the leptin receptor, which results in not only an obesity model but also has characteristics of insulin resistance, hyperinsulinemia, hypertriglyceridemia, hypercholesterolemia. Thus the effects on contractility that were observed could be due to these other characteristics.

Some limitations affecting the current study must be considered. First, the absence of nerve and blood supply in ex vivo, despite being advantageous for some investigations, also raises questions as to whether urethral functional deficits could be attributed to compromised innervation, failures at the neuromuscular junction, or issues related to altered metabolism or circulation. This is especially relevant when interpreting the effects of muscle fatigue. Second, the urethral sphincter ring was composed of both smooth and striated muscle. Due to the small size of urethral ring used in experiments, we did not separate smooth muscle from striated muscle of urethra sphincter. So the contractile response of the smooth muscle component can not be completely excluded in this study; even though we applied the EFS with low frequency and duration (1 Hz and 0.2 ms) that have been shown to evoke very low contractile response in smooth muscle.[30,31] Third, due to lack of molecular production and pathological study, a detailed discussion on the potential mechanisms should be limited. To elucidate these potential relationships, deeper investigation into the basic pathophysiology of SUI in obese females is clearly needed to improve our understanding of the causes and to allow for development of targeted therapeutic approaches.

5 | CONCLUSIONS

This novel study showed that obese female rats had significantly impaired contractile properties of urethral sphincter, suggesting urethral dysfunction could be an important contributor to SUI in obesity.

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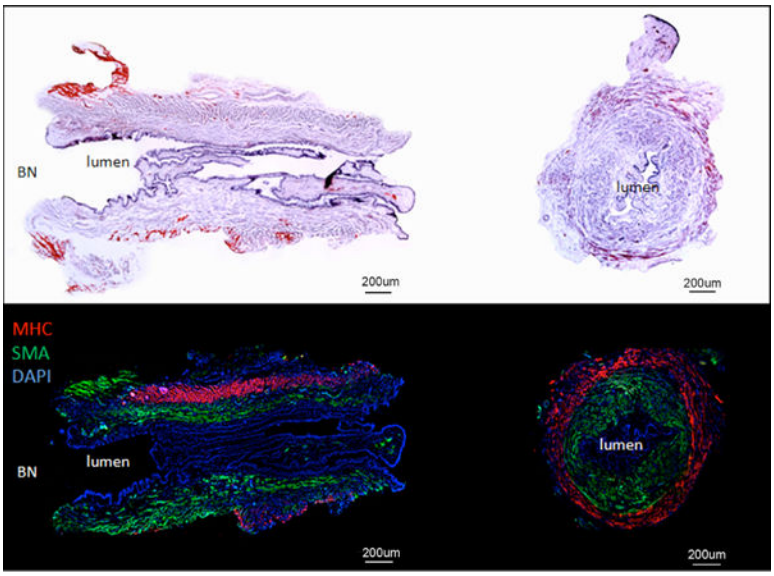

FIGURE 1.

Histological examinations of the female rat urethra used in experiment. Representative morphology showed the corresponding location of the longitudinal and cross sections of isolated urethra using H&E staining (upper panel) and immunohistochemical staining (lower panel). The immunohistochemical staining clearly showed that the entire striated muscle (MHC) was included in the urethra ring used in the experiment

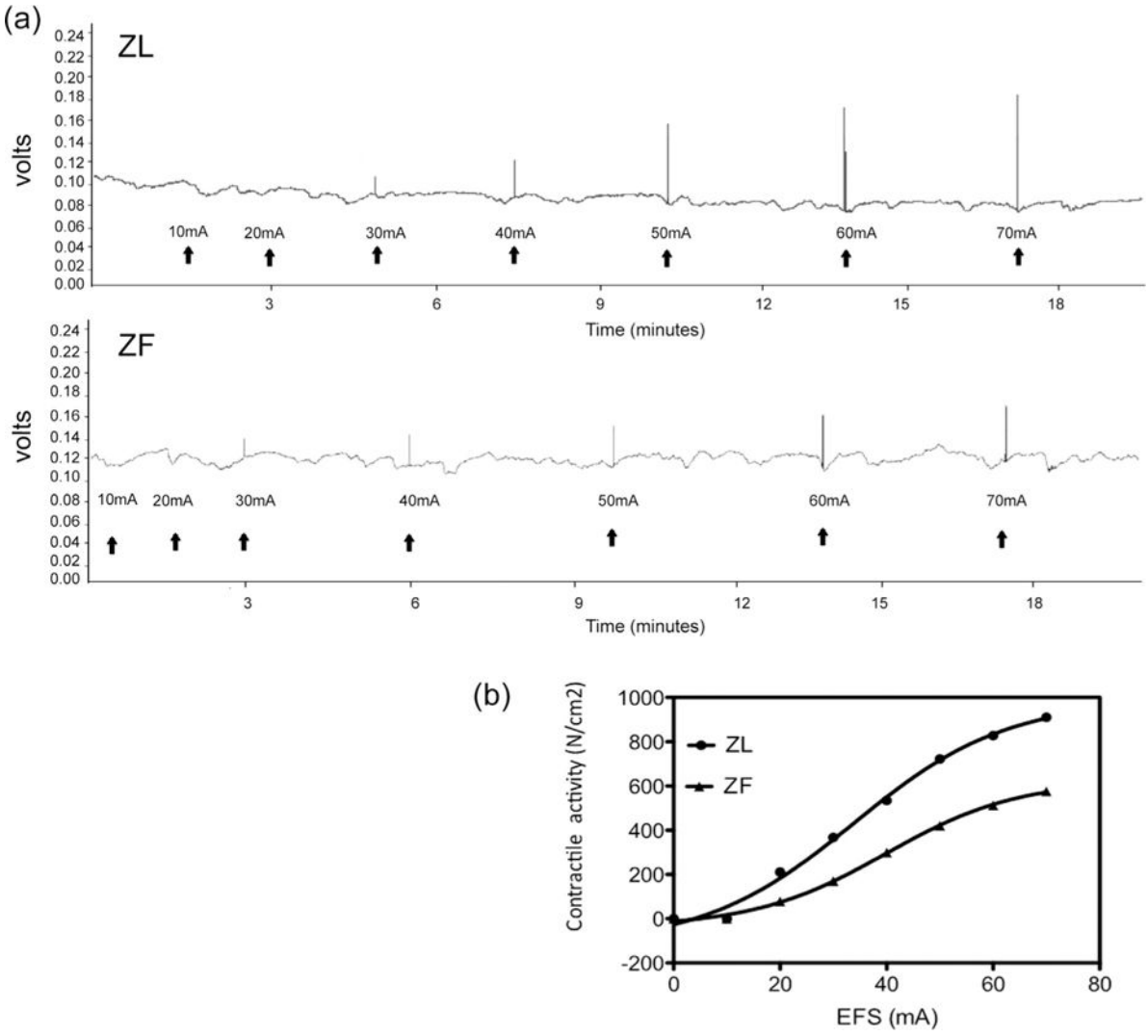

FIGURE 2.

Contractions of urethral striated muscles induced by single-pulse electrical field stimulations (EFS) in female rats. (a) Representative graph showing the individual contractions of a force versus different voltage (from 10 to 70 mA) and the relationship with response to EFS in both groups. (b) Representative graph of the plotted curve of absolute muscle contractile activity (MCA) resulting from individual contractions. Compared to ZL controls, ZF rats had significantly impaired MCA and a lower percentage of MCA increment from baseline ($n = 10$, $P < 0.05$). Arrow: electric filed stimulation point

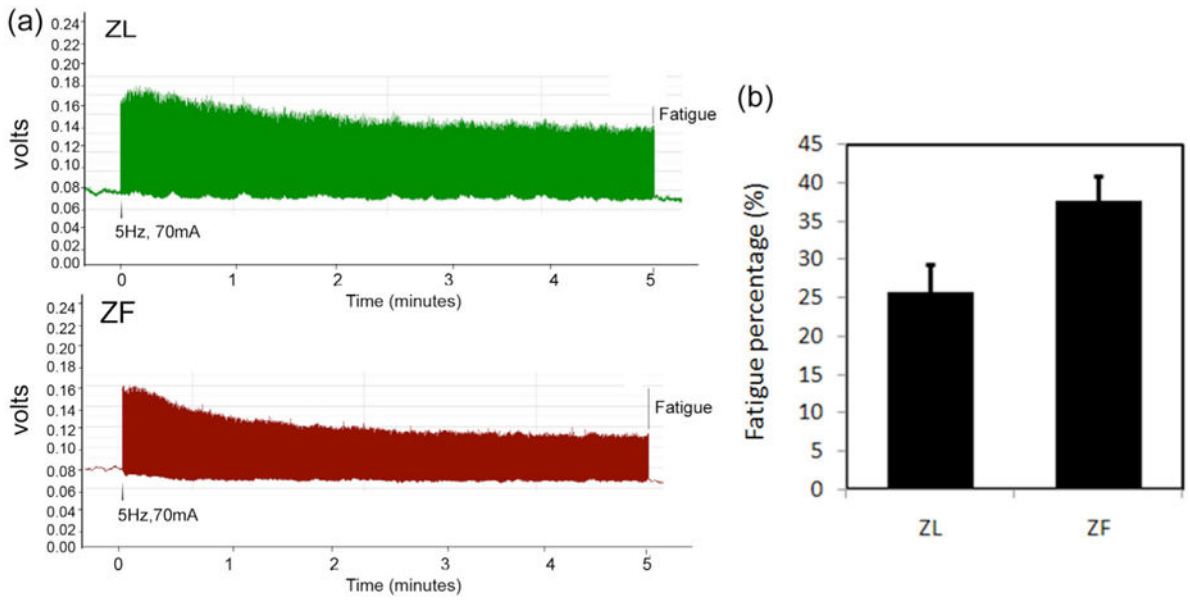

FIGURE 3.

Fatiguing stimulation test induced by repeated multi-pulse EFS in female rats. (a) Representative graph showing the typical decline fatiguing profile of the urethral striated muscle in both groups. (b) Compared to ZL controls, ZF rats presented early fatiguing of MCA and also had significantly greater percentage of MCA decline from baseline ($n = 10$, $P < 0.05$)

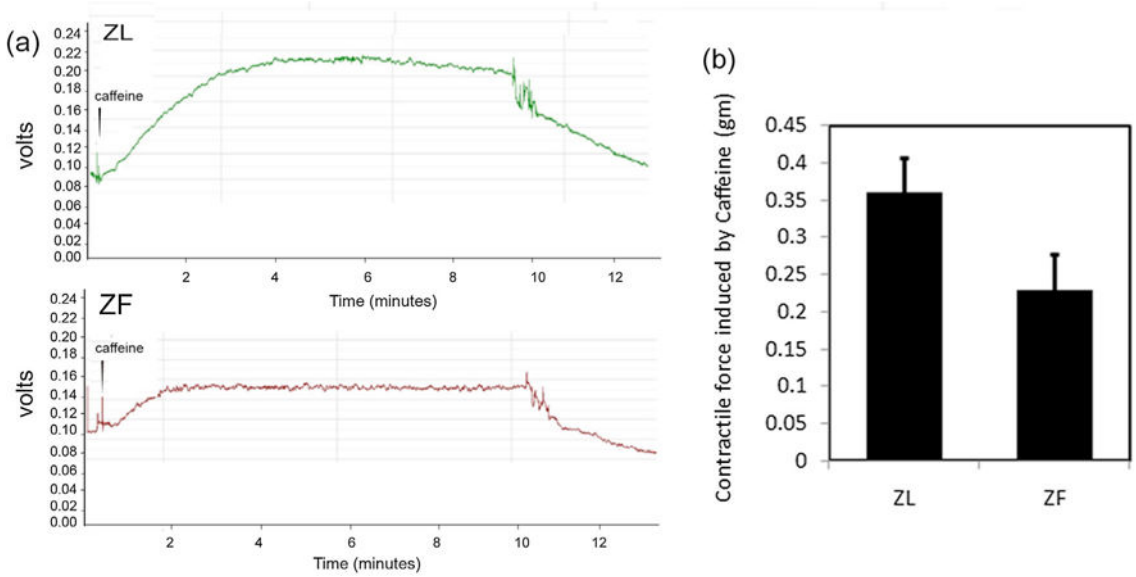

FIGURE 4.

Urethral striated muscle contractile activity (MCA) stimulated by caffeine. (a) Representative graph showing the MCA profile stimulated by caffeine in both groups. (b) Compared to ZL controls, ZF rats had significantly impaired maximal MCA after caffeine was administrated ($n = 10$, $P < 0.05$)