Histomorphometric analysis of a rat bladder after electrical stimulation

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Summary

Objective: We sought to analyze the effects of electrical stimulation (ES) of the pelvic floor on the bladder of rats. **Methods:** Forty rats were studied and divided into the following groups: GI (control group) - did not receive ES, GII (placebo) - did not receive ES but had an electrode inserted into the vagina; GIII - underwent six sessions of ES of the pelvic floor and GIV - rats that underwent 12 sessions of ES. Subsequently, the bladder was removed and the epithelium, muscle and blood vessels were analyzed. **Results:** The muscle wall in GIV had increased thickness when compared to other groups. Further, the number of blood vessels was similar in GIII and GIV, which was higher than that found in GI and GII. Finally, there was an increase in the relative percentage of muscle fibers in relation to collagen for GIV compared to GI. **Conclusion:** After 12 sessions of ES in rats the muscle layer, the number of blood vessels and the relative percentage of muscle fibers were increased.

Keywords: Urinary bladder; transcutaneous electric nerve stimulation; morphology.

Resumo

Análise histomorfométrica da bexiga de ratos após eletroestimulação

Objetivo: Analisar os efeitos da eletroestimulação (ES) de assoalho pélvico na bexiga de ratas. **Métodos:** 40 ratas foram estudadas e divididas nos seguintes grupos: GI (Grupo controle) - não receberam ES GII (placebo) - não foram submetidas a ES mas possuíam um eletrodo introduzido dentro da vagina; GIII - submetidas a seis sessões de ES do assoalho pélvico e GIV - ratas que receberam 12 sessões de ES. Posteriormente, a bexiga foi removida e analisou-se o seu epitélio, musculatura e os vasos sanguíneos. **Resultados:** Observou-se aumento na espessura da parede muscular em GIV em relação aos demais grupos. Encontramos, ainda, que o número de vasos sanguíneos foi similar em GIII e GIV, sendo maior ao observado em GI e GII. Por fim, houve aumento na porcentagem relativa de fibras musculares em relação ao colágeno em GIV quando comparada à GI. **Conclusão:** Após 12 sessões de ES em ratas houve aumento da camada muscular, número de vasos e da porcentagem relativa de fibras musculares.

Unitermos: Bexiga urinária; estimulação elétrica nervosa transcutânea; morfologia.

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Conflit of interest: None.

Introduction

Urinary incontinence may cause significant social distress as well as medical concern. It constitutes an important public health problem and has a high impact on the quality of life[1,2,3]. Risk factors for urinary incontinence include obesity, hypoestrogenism, genetic factors, cough, previous gynecological surgeries, age, and parity[4].

Urinary continence is achieved by the interactions of mechanisms responsible for closing the urethra[5]. Currently, literature suggests that urinary continence is also related to integrity of the pelvic floor muscles[6-9]. Andersson *et al.*[10] observed muscle arrangements similar to those in humans when studying muscle functional responses in the urethra of animals. The periurethral and pelvic floor muscles are comprised of type I (slow-contracting) and type II (fast-contracting) fibers. The type I fibers are able to maintain the tonus and the type II are related to an additional occlusion response during intra-abdominal pressure increase[11,12].

The large and numerous periurethral vessels efficiently meet blood requirements of the urethra. They form a structure similar to the penian *corpus spongiosum* for erectile function wherein intravascular pressure can be mechanically transmitted to the urethra for its closing and maintenance of continency[5, 13]. The connective tissue is another important factor to maintain continence, because its mechanical function is to resist tension force[14]. This property arises from the collagen fibers, important to maintain the elastic proprieties of the extracellular matrix.

Treatment for urinary incontinency can be divided into clinical and surgical approaches. The surgical approach was the first choice of treatment until 1980; thereafter, clinical treatment with use of physiotherapeutic techniques gained interest[16, 17]. The Department of Health and Human Services of the United States of America considers that treatment of the different forms of urinary incontinence must be undertaken with less invasive procedures. Treatment includes perineal exercises, vaginal cones and electrical stimulation[17, 18, 19]. Perineal exercise is, according to physiotherapy the best scientific approach for treating women with stress urinary incontinence. Introduced by Arnold Kegel in 1948, he asked patients to practice 300-400 contractions of the pelvic floor. Later in 1956, the therapy was changed and the concept of progressive resistance in treatment of stress urinary incontinence was introduced. He obtained a cure rate of above 70%[20]. The vaginal cones are simple and practical to identify and strengthen the pelvic floor muscles, using the principles of biofeedback. In 1985 Plevnik showed that patients can learn to contract the pelvic floor muscles through retention of vaginal cones with increasing weights[21]. In view of the neuromuscular etiology of the most prevalent types of urinary incontinence, i.e., stress urinary incontinence and overactive bladder, it can be stated that electrical stimulation of the pelvic floor is effective in restoring muscle and nerve functions. Thus, this treatment modality is effective in both disorders[22, 23, 24].

Castro *et al.*[25] showed that the closing pressure and functional length of the urethra do not change with electrical stimulation in women with stress urinary incontinence; however, vesical capacity significantly increased after treatment. Electrical stimulation (ES) of the pelvic floor has proven to be an efficient form of treatment for stress urinary incontinence and hyperactive bladder conditions[26, 27]. Currently, ES is the most frequently used treatment for urinary incontinence. Muscle electrical stimulation at a low frequency aims to facilitate muscle contraction with "on" and "off" cycles that have been shown to avoid the muscle fatigue of continuous contraction[28, 29].

ES is able to increase intraurethral pressure and reestablish neuromuscular connections. These changes can improve the vascular area and muscular function, thereby promoting muscle hypertrophy and a better resistance to fatigue[30, 31, 32]. Since there is a lack of literature examining histological effects of ES, this paper was designed to study the effects of electrical stimulation on the bladder of female rats.

Methods

This study was performed in the Urogynecology and Vaginal Surgery Division of the Gynecology and Morphology Departments of the *Universidade Federal de São Paulo* (UNIFESP-EPM). Forty adult (60-90 days old) female virgin rats (*Rattus norvegicus albinus,* Rodentia, Mammalia) were obtained from our animal facilities at the Center for Developement of Experimental Models for Medicine and Biology (*Centro de Desenvolvimento de Modelos Experimentais em Medicina e Biologi*a - CEDEME) of UNIFESP-EPM. The project was approved by the local Ethics Committee (Protocol no. 088/03).

Animals were housed (5 per cage) on a 12:12 h light/dark cycle. Pelleted rat ration (Labina-Purina, São Paulo, Brazil) and water were available *ad libitum*. After a 2-week adaptation period, rats were divided into four groups: GI (Control group, n = 10) rats received no electrical stimulation; GII (Placebo group, n = 10) rats received no electrical stimulation but had an endovaginal electrode; GIII (Group III, n = 10) rats were submitted to six sessions of endovaginal electrical stimulation; GIV (Group IV, n = 10) rats were submitted to 12 sessions of endovaginal electrical stimulation. The electrical stimulation of the pelvic floor was performed with a specific stimulator device *Vagitonus Stimulatorâ*, Viotti S/A, São Paulo, Brazil)" using a special 2-mm diameter probe. Pulse stimulations of 1 ms duration were applied at a low frequency (50Hz) with an intensity of 20 ma in accordance to previous protocols[33].

The animals were submitted to two 15 minute sessions per week after being anesthetized with xylazine (5 mg/kg) and ketamine (5 mg/kg) diluted in saline solution.

After 8 days of treatment with electrical stimulation, the animals were sacrificed with a lethal injection of anesthesia. We performed a longitudinal incision to remove the uterus, bladder and urethra at once. The bladder was opened by a transversal axe and fixed in Bouin's liquid for 24 hours. The material was dehydrated in graded concentrations of ethanol and embedded in paraffin. From each bladder, 4-µm sections were obtained and stained with hematoxylin and eosin (HE). Picrosirus red was used to evaluate collagen as well as muscular percentage[34].

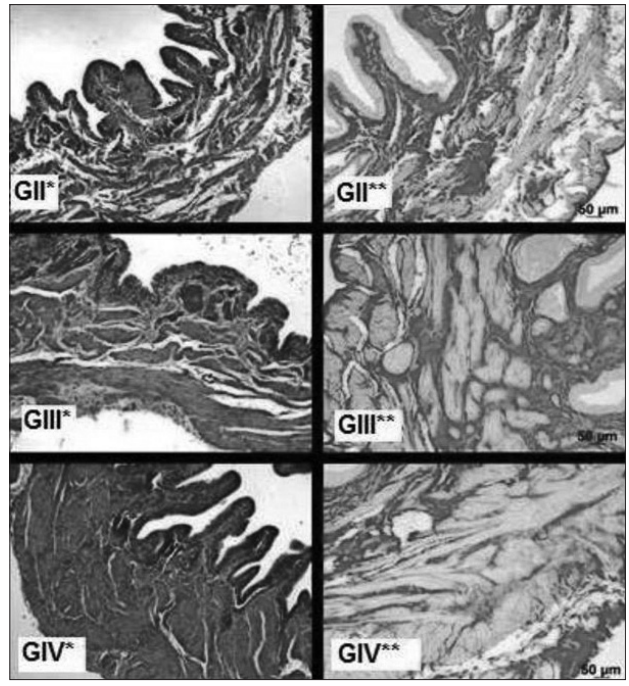
The slides were systematically scanned in a microscope using 40 to 400 X magnification for morphological description. Morphometric analysis of the muscle fibers and blood vessels of the bladder was studied after image capture with a light microscope using 400 X magnification (Axiolab Standart 2.0, Carl Zeiss). The microscope was coupled to a video camera (AxionCam, Carl Zeiss) and the software, Axion Vision 4.3, was utilized for measurement of the epithelium, muscle layer thickness and number of blood vessels. We obtained the median of ten measurements for each 1000 mm^2 slide. The percentages of collagen, muscle and light space in the images were obtained from cuts stained by picrosirius red and analyzed using IMAGELAB (SOFTIUM Informatics LTDA).

Data were expressed as the means ± standard deviation (SD). Values were compared between groups using ANOVA for multiple comparisons. Statistical analyses were performed using a standard computer software package (GraphPad Prisma, GraphPad Software, San Diego, CA, USA). p -values of less than 0.05 were considered statistically significant.

Results

We observed that GI and GII had similar, well-characterized transition epithelia. Under the epithelium, the *lamina propria* was seen to have collagen fibers, blood vessels and conjunctive cells (e.g., fibroblast, mastocytes and leukocytes). The muscle layer was comprised mainly of smooth muscle fibers with different orientations. Between fibers we observed spaces with blood vessels and conjunctive tissue. Externally, the bladder was covered with a simple squamous epithelium (mesothelium) in some areas (Figure 1).

Figure 1



GIII had histological characteristics similar to those of GI and GII with a transition epithelium and *lamina propria* underneath. The muscle layer presented muscle fibers larger than those in the others groups with light space and collagens fibers (Figure 1). GIII had a thicker bladder wall than the other groups. Additionally, the mucosa was covered with a thicker transition epithelium. Underneath, we observed a *lamina propria* rich in collagen fibers with many blood vessels. The muscle layer was thick with smooth muscle fibers and large nuclei. We could detect rare light spaces between the muscle fibers. The external portion was covered with squamous cells comprising a mesothelium (Figure 1).

The histomorphometric results were assessed by measuring the transition epithelium, muscle layer and number of blood vessels (Table 1). The muscle layer was larger in GIV than the other groups ($p < 0.05$). The mean number of blood vessels (area of 10^3 mm^2) differed significantly between groups. Values in GIII were similar to those in GIV and larger than those in GII and

Table 1 – Mean and standard deviation of the urinary epithelium, muscle layer and blood vessel number in the bladder of all groups

	GROUPS			
	GI	GII	GIII	GIV
Epithelium (mm)	16.20 ± 4.80	15.10 ± 3.40	17.32 ± 6.10	16.03 ± 5.90
Muscle layer (mm)	528.75 ± 156.10	491.79 ± 80.46	509.89 ± 165.16	674.25 ± 124.08*
Blood vessels (10^3um^2)	0.196 ± 0.27	0.1354 ± 0.065	0.5342 ± 0.19**	0.6245± 0.66**

GI (control group), did not receive electrical stimulation (ES); GII (placebo), were not submitted to ES but had an electrode inserted in the vagina; GIII, underwent six sessions of pelvic floor ES; GIV, rats that received 12 sessions of ES; *, **p < 0.05

GI ($P < 0.05$). There were no significant differences between groups with regard to percentage of collagen fibers (53% in GI, 42.9% in GII, 51.8% in GIII, and 45.6% in GIV). The percentage of muscle fibers was measured in all the groups (45.44% in GI, 48.31% in GII, 48.18% in GIII, and 53.87% in GIV). There was a significant difference only between GIV and GI ($p < 0.05$) (Table 2).

Discussion

It is well known that factors such as pregnancy and hypoestrogenism play a role in urinary incontinence. Physiological and anatomical alterations that occur with age are an important cause of urinary tract symptoms in women[35]. Urinary incontinence is an common symptom during the post-menopausal period, when it is a result of decreased estrogen and collagen density. Thus, an understanding of these facts can improve continence[4, 35,36].

The electrical stimulation technique used in this study is a well known clinical treatment. However, there is almost no work in literature on its mechanism of action. When used in humans, electrical stimulation can help maintain the strength of the muscular pelvic floor[25, 37]. Duration of treatment is typically three months, with sessions occurring twice a week[25].

Castro *et al.*[37] concluded that electrical stimulation, pelvic floor exercises and vaginal cones are equally effective treatments in women with urodynamic stress urinary incontinence. Some authors have shown that estrogen treatment can improve urinary continence in ovariectomized female rats. This hormone causes an increase of blood vessels, collagen, and smooth muscle [14, 39,40,41,42].

Our study has demonstrated that the effects of electrical stimulation were similar to those of estrogen with regard to the increase in blood vessels and muscle thickness. Roles of estrogen in increasing glycoprotein and acting on vascular endothelial growth factor (VEGF) which increase the number of blood capillaries are well known [43]. We believe that electrical stimulation may increase secretion of glycoprotein and the number of blood vessels.

Arruda *et al.*[37] have shown that 76% of patients with a hyperactive bladder were cured or had improved urinary continence after electrical stimulation. The detrusor muscle is stimulated by sympathetic and parasympathetic nerves via specific neurotransmitters. The parasympathetic system is important for the emptying and the sympathetic for filling of the bladder, which occurs due to membrane depolarization of smooth muscle cells[44]. Electrical stimulation depolarizes the cell membrane in a continuous manner.

In a recent prospective, randomized controlled trial statistically significant improvement in some urodynamic parameters was observed, voiding diary parameters, urgency severity, incontinence Impact Questionnaire short form and Beck Depression Inventory scores at the end of ES[24]. Nowadays, electrical stimulation of the tibial nerve appears as an excellent therapeutic option for urinary incontinence with level I evidence[45].

We believe that electrical stimulation can increase the amount of actin, myosin and intermediate filaments of the muscle layer, thereby producing an increase in the layer's thickness[45]. The group that received more stimulation exhibited a greater response in a dose-dependent manner. This study describes important changes in both thickness and blood vessel number. Moreover, we demonstrated the improvement of symptoms with electrical stimulation. Of course, more research in this field is required.

Conclusion

After twelve sessions of electrical stimulation in rats there was an increase in the thickness of the muscle layer, number of blood vessels and percentage of muscle fibers.

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Table 2 – Percentage of collagen, muscle, and light space for all groups

	GROUPS			
	GI	GII	GIII	GIV
Collagen (%)	53.02 ± 5.04	42.91 ± 2.92	51.8 ± 5.89	45.62 ± 11.5
Muscle (%)	45.44 ± 4.72	48.31 ±3.58	48.18 ± 4.42	53.87 ± 7.80*

GI (control group), did not receive electrical stimulation (ES); GII (placebo), were not submitted to ES but had an electrode inserted in the vagina; GIII, underwent six sessions of the pelvic floor ES; GIV, rats that received 12 sessions of ES; * $p < 0.05$

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