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Alterações morfológicas das fibras tipos I e II do músculo estriado uretral de ratas prenhes diabéticas

Gabriela Marini

Dissertação apresentada ao Programa de Pós-Graduação em Ginecologia, Obstetrícia e Mastologia, da Faculdade de Medicina de Botucatu - UNESP, para obtenção do título de Mestre.

Orientadora: Profa. Titular Marilza Vieira Cunha Rudge

Co-orientadora: Profa. Dra. Angélica Mércia Pascon Barbosa

Co-orientadora: Profa. Dra. Selma Maria Michelin Matheus

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Epígrafe

“**Perder alguém querido**,

Palavras não explicam a morte de alguém querido. Sabem disso o pai, a mãe, os filhos, os irmãos, o marido e a mulher, e os amigos de verdade.

Quando o outro morre, parte do mistério da vida vai com ele. A parte que fica torna-se mais intrigante. Descobrimos a relação profunda entre a vida e a morte, quando alguém que era a razão de nossa vida vai-se embora.

Para onde? Para quem? Está me ouvindo? A gente vai se ver de novo? Como será nosso reencontro? Acabou-se para sempre, ou ela apenas foi antes? Por que agora? Por que deste jeito? As perguntas insistem em aparecer e as respostas não parecem claras. Dói, dói, dói.

Um dia nos veremos de novo. Enquanto este dia não vem, quem eu amo e se foi. Para ela, a vida tem agora, outra dimensão. Alcançou o infinito.

Por enquanto fica apenas o mistério. Alguém que não sabemos por que entrou tão cheio em nossas vidas, fechou os olhos e foi-se embora. Quem ama de verdade não crê que se acabou. A vida é uma só. Começa aqui no tempo e continua depois, na ausência de tempo e limite.

Alguém a quem amamos se tornou eterno. E essa pessoa já sabe quem e como Deus é. E também sabe o porquê de sua partida. Se ela está no céu então alguém, além de Deus e de Jesus, se importa comigo. Definitivamente, não estamos sozinhos por mais que doa a solidão de havê-lo perdido.

Mas é apenas por pouco tempo

Quem amou aqui, sem dúvida, se reencontra no infinito.

Ninguém ama aquilo que não conhece

Mas ninguém esquece daquilo que ama.

Quando tudo falha é tempo de confiar!”

Padre Zezinho

Sumário

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Filipenses 4:4

Capítulo 1

Diabetes in pregnancy and urinary incontinence: a little acknowledged association

Gabriela Marini[1], Angélica Mércia Pascon Barbosa[1], Débora Cristina Damasceno[1], Rodrigo de Aquino Castro[2], Selma Maria Michelin Matheus[3], Marilza Vieira Cunha Rudge[1] *

[1] Experimental Research Laboratory , Department of Gynecology and Obstetrics - Botucatu Medical School, UNESP - Univ Estadual Paulista, São Paulo, Brazil.

[2]Department of Gynecology, São Paulo Federal University (UNIFESP), São Paulo, Brazil.

[3]Department of Anatomy, Botucatu Biosciences Institute, UNESP - Univ Estadual Paulista, São Paulo, Brazil.

* *Correspondence*: Marilza Vieira Cunha Rudge. Experimental Research Laboratory, Department of Gynecology and Obstetrics, Botucatu Medical School, UNESP -Univ Estadual Paulista – CEP 18618-000 - Botucatu, São Paulo, Brazil

Este capítulo foi redigido de acordo com as normas de publicação da revista **Diabetology & Metabolic Syndrome**, para a qual foi submetido.

Abstract

Urinary incontinence (UI) in women is defined as any involuntary urine loss. It is a frequent condition of high economic cost to the government that also results in women's physical, psychological and social damage and impaired quality of life. Various risk factors are involved in UI development; however, association with Diabetes *mellitus* (DM) is of great interest at present. DM affects multiple organ systems, including the urinary system in approximately 52% of diabetic patients and in those showing only hyperglycemia; however, the association between gestational DM and UI has not been fully explained. Health care professionals must be attentive to this new parameter and attempt to analyze it in more detail so that prophylactic and therapeutic measures can be established. It is necessary to delineate the chronology of the relationship between gestational Diabetes *mellitus* and vesical complications, the relationship between controlled diabetes and the incidence of incontinence as well as effective treatment modalities for diabetic patients with symptoms in the lower urinary tract.

Key words: diabetes, pregnancy, urinary incontinence

Introduction

Urinary incontinence (UI) in women is defined as any involuntary urine loss [1]. It is a frequent condition of high economic cost to the government that also results in women's physical, psychological and social damage and impaired quality of life [2, 3].

The world epidemic of obesity and type-2 diabetes has significant implication in the occurrence of UI in women [4]. However, the mechanisms by which diabetes contributes to the UI development and severity are not well defined.

Diabetes is related to muscular strength and physical function impairment, and it may reflect the connection between the muscle's metabolic and mechanic functions. There is a temporal relation between Diabetes *mellitus* (DM) diagnosis and the subsequent development of muscular weakness associated with complications such as diabetic amyotrophy. Hyperglycemia may affect the contractile function and strength generation in the muscle [5].

In 2006, the association of Gestational Diabetes *mellitus* (GDM) with UI increases and muscular dysfunction on the pelvic floor two years after gestation became clear. Pregnant women with GDM showed 50.8% of UI in pregnancy as compared to 31.6% in non-diabetic individuals ($p<0.05$). The presence of GDM increased not only IU occurrence two years after childbirth, but it was also associated with UI in pregnancy [6]. Such association is little acknowledged in the literature because there are still other concerns to be solved regarding the diabetes-pregnancy binomial.

Historical development of GDM

The 20th century witnessed the change in pregnant women's follow-up. At the beginning of that century diabetic women died; they later became infertile and rarely had successful at-term pregnancies. The advent of insulin in 1922 abolished maternal death and, over the past century, all efforts were made to improve perinatal results.

The first concern was the moment of childbirth and, later, the perinatal complications resulting from intrauterine hyperglycemia, such a fetal macrosomy [7]. White's clinical classification [8] established a direct relation between the severity of maternal clinical conditions and the moment of delivery, and it was fundamental in reducing perinatal death. As a result, there was significant increase in cesarean section rates in the population [9].

Despite all the present efforts, perinatal death rates, congenital malformation cases excluded, is slightly higher than those found for the non-diabetic population [10]. At the moment, the literature attempts to solve the problem of fetal malformations and of the large obesity and diabetes epidemic around the world which has resulted in the increase of pregnancies complicated by diabetes. It can be imagined that, in this century, the concern regarding this binomial will be focused on preventing the occurrence of fetal malformation, finding more refined and earlier diagnostic methods as well as on furthering the knowledge on more subtle GDM outcomes in the maternal organism.

Although diabetes is the most common medical complication in pregnancy, the association between GDM and UI is not reported in obstetric textbooks. The recommended classification is based on the quality of maternal metabolic control and emphasizes that the compromising of target organs, such as the kidneys, eyes and

heart, has a significant effect on pregnancy outcomes. That classification does not make any reference to the compromising of the lower urinary tract in diabetic pregnant women or to whether this may be an aggravating factor in maternal and perinatal prognosis.

Diabetes in pregnancy

DM is one of the most common endocrine disorders, and it affects approximately 7% of the world population and 50% of diabetic individuals are not aware of their diagnosis [11]. Diabetic pregnant women can be separated into two large groups: those who already had the diabetes diagnosis prior to pregnancy (clinical diabetic women) and those who are diagnosed during their pregnancies (gestational diabetic women). It is estimated that 10% of them have clinical diabetes and 90% gestational diabetes [7].

The total number of individuals with diabetes is expected to increase from 171 million in 2000 to 366 million in 2030. Its expansion follows population growth and ageing, urbanization as well as obesity and sedentariness increase [12]. Such population growth in the diabetic population worldwide will not only increase diabetes occurrence in pregnancy, but it will also change the proportion of clinical and gestational diabetic women.

Diabetes *mellitus* and urinary incontinence

DM affects multiple organ systems, including the urinary system in approximately 52% of diabetic patients and in those showing only hyperglycemia. Various epidemiological studies have observed increased risk (50 to 200% more commonly) for UI in women with type-2 as compared to those with normal glucose

levels [13]. The risk factors involved in UI development are many; however, the association with diabetes is of great interest at present.

Diabetes, waist circumference, parity and low social support are associated with increased prevalence of stress urinary incontinence (SUI) whereas a high body mass index and impaired health are associated with UI incidence [14].

UI is also common among women with DM 1, and risk factors including old age, weight increase and previous urinary infection are important. Weight reduction and infection treatment can prevent UI or reduce its severity [15].

UI prevalence in a group of 1,585 women older than 20 years was of 49.5%. UI was significantly associated with older age, poor education, recurrent urinary-tract infection, Diabetes *mellitus*, history of nocturnal enuresis in childhood, diuretic medication and BMI [3]. It was also characterized as more severe in women with DM [16].

Urge UI was more prevalent in non-diabetic women whereas mixed UI and SUI were more prevalent in diabetic women. Of the diabetic women, 41% reported UI, and DM was an independent determinant factor of UI [17].

UI was reported by 65% of the women with DM 1. Of these, 40% were very annoyed by their incontinence, and 9% believed that it did not affect their daily activities. The prevalence of weekly urge incontinence was twofold in women with DM 1 as compared to that of women without diabetes. Additionally, UI prevalence was higher than that of neuropathy, retinopathy and nephropathy. These findings point out the importance of tracking urinary incontinence among women with DM 1 [18].

Brown *et al.,* [13] found high UI incidence in women with DM 2 or with hyperglycemia when compared to women with normal glucose levels.

This literature review clearly shows that the association between diabetes and UI has been acknowledged. Nevertheless, the clinical meaning of UI, its short- and long-term outcomes and the need or not for treatment and prophylaxis has not been established.

Diabetes in pregnancy and UI

The association between GDM and increased UI prevalence and muscular dysfunction of the pelvic floor has been clearly shown by Barbosa,[6] who concluded that the prevalence of gestational UI two years after childbirth was significantly higher in women with GDM than among normoglycemic pregnant women. Multivariate analysis has shown GDM to be an independent risk factor for the occurrence of gestational UI.

Kim *et al.,* [19] also observed that 49% of the women with GDM reported frequent incontinence during pregnancy, and 28% reported that such UI affected their daily activities. They concluded that SUI is common among women with GDM, and it does not seem to be associated with physical activity levels or body mass index.

DM was also associated with vesical dysfunction, such as sensory abnormalities that result in vesical sensitivity impairment, increased complacency and increased residual volume; UI and sexual dysfunction in women, such as inhibited desire, pain during intercourse and inadequate lubrication [20].

These reports on the association of GDM with UI are relatively recent in the literature, little acknowledged and little valued by medical professionals. There are no reports in the literature on the clinical maternal meaning of such UI in pregnancy and nor on the possible correlations between its occurrence and maternal and perinatal outcomes.

Experimental models

Experimental severe diabetes induction is well established, including in pregnancy [21], and many studies have confirmed its effects on the lower urinary tract of animal models.

Diabetic rats with higher glycemia than 300mg/dL (6-8 weeks) showed significantly increased vesical capacity and increased intercontraction intervals, extensive damage in the external urethral sphincter (EUE), atrophy in the urethral vaginal septum and increased collagen deposition between the striated muscles. UI was more severe, and the recovery of the damages generated by vaginal distention was delayed in the diabetic group. The authors suggest that diabete is related to the accumulation of free radicals and ischemia, which can interact or be independent factors to generate dysfunctions in the lower urinary tract [22].

Diabetes caused effects on the bladder and urethra of rats 06 weeks after induction, such as decreased vesical sensitivity, increased vesical capacity, increased residual volume and detrusor contractility impairment. After 20 weeks, an atrophy was found in the EUE, which was related to the polyneuropathy found in DM [23]. Significant decrease in the skeletal muscle mass of STZ-induced rats (example of diabetic myopathy) was also observed in another study [24].

Diabetes is also associated with a reduction in the capillarization of the skeletal muscle and a deregulation in the angiogenesis route in the quadriceps muscle of rats with severe diabetes (3 to 5 weeks) [25].

However, in more recent studies, pregnancy and caesarean sections did not induce alterations in the number of collagen, muscle, elastic and nerve fibers. But vaginal birth and simulation of delivery trauma reduced muscle and nerve fibers and increased collagen and elastic fibers [26].

Studies on diabetic pregnant rats have not been found.

Conclusions

Despite the high UI prevalence in diabetic pregnant and non-pregnant women, many of them do not report their incontinence to clinicians, and those who do are given the simplest explanation attributed to polyuria caused by DM itself. Such knowledge has been recently developed in this century, and the association between GDM and UI and its relevance has not yet been established. Science and medicine move as follows: firstly, a phenomenon is observed. Later, its actual importance is analyzed and then the need for treatment and prophylaxis is evaluated. The phenomenon is confirmed. Its importance must be studied in experimental models and in clinical observations during pregnancy by evaluating its maternal and perinatal outcomes. Health care professionals must be attentive to this new parameter and seek to analyze it in more detail so that prophylactic and therapeutic measures can be established.

It is necessary to delineate the chronology of the relationship between GDM and vesical complications, the relationship between controlled diabetes and the incidence of incontinence as well as effective treatment modalities for diabetic patients with symptoms in the lower urinary tract.

List of abbreviations

UI (urinary incontinence), DM (Diabetes *mellitu*s), GDM (Gestational Diabetes *mellitus*), SUI (stress urinary incontinence), EUE (external urethral sphincter).

Competing interests

The authors declare that no competing interests exist.

Authors' contributions

All authors have participated in the manuscript's design and drafting. All authors have participated in the study's review of the data shown and they have read and approved of the final manuscript version.

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Bom mesmo é ir a luta com determinação, abraçar a vida com paixão, perder com classe e vencer com ousadia, pois o triunfo pertence a quem se atreve. A vida é muito para ser insignificante.

Charles Chaplin

Capítulo 2

Morphological changes in muscle mass and in fast-to-slow fiber profile in urethral striated muscle fibers of diabetic pregnant rats.

Gabriela Marini[1], Angélica Mércia Pascon Barbosa[1], Débora Cristina Damasceno[1], Rodrigo de Aquino Castro[2], Selma Maria Michelin Matheus[3], Jorge Milhem Haddad [4], Suely Kazue Nagahashi Marie[5], Manoel João Batista Castello Girão[2], Marilza Vieira Cunha Rudge[1] *

[1] Experimental Research Laboratory, Department of Gynecology and Obstetrics, Botucatu Medical School, UNESP - Univ Estadual Paulista, São Paulo, Brazil.

[2]Department of Gynecology, São Paulo Federal University (UNIFESP), São Paulo, Brazil.

[3]Department of Anatomy, Botucatu Biosciences Institute, UNESP - Univ Estadual Paulista, São Paulo, Brazil.

[4] Department of Gynecology, University of São Paulo (USP), São Paulo, Brazil.

[5]Department of Neurology, Clínicas Hospital, School of Medicine, University of São Paulo (USP), São Paulo SP, Brazil.

* *Correspondence*: Marilza Vieira Cunha Rudge. Experimental Research Laboratory, Department of Gynecology and Obstetrics, Botucatu Medical School, UNESP - Univ Estadual Paulista CEP 18618-000 - Botucatu, São Paulo, Brazil. E-mail address: marilzarudge@ig.com.br

Este capítulo foi redigido de acordo com as normas de publicação da revista **Internacional Urogynecology Journal**, para a qual será submetido.

Abstract

The aim of this study was to evaluate the morphological alterations of the urethral striated muscle and type I and II muscle fibers in diabetic pregnant rats that underwent cesarean section. Twenty female Wistar rats were distributed in four experimental groups of five rats: virgin (control), pregnant (control), diabetic virgin (control), and diabetic pregnant. Diabetes was induced by streptozotocin administration. The rats were lethally anesthetized and the urethra and vagina were extracted as a unit. Cryostat sections of 6-µm thickness were cut and stained with hematoxilin-eosin and immunohistochemical procedures were performed and subjected to morphological and semi-quantitative analysis. In comparison with muscle from the three control groups, urethral striated muscle from diabetic pregnant rats presents with the following variations: thinning and atrophy, disorganization and disruption associated with co-localization of fast and slow fibers and a steady decrease in the proportion of fast to slow fibers. Our results indicate that diabetes and pregnancy impair the urethral striated muscle and alter the distribution of its fiber types.

Keywords: diabetes, pregnancy, striated muscle fiber, urethra

Introduction

Diabetes *mellitus* (DM) during pregnancy was associated with high levels of urinary incontinence (UI) and pelvic floor muscle dysfunction two years after cesarean section. The risk factors for pelvic floor muscle dysfunction among these women were related to high newborn weight and high maternal weight gain during pregnancy as a result of Gestational Diabetes *mellitus* (GDM). Furthermore, the risk factors for UI were indirectly influenced by GDM and pelvic floor muscle dysfunction. This framework confirms an association between GDM and subsequent pelvic floor muscle dysfunction two years following cesarean section [1] .

Data from an epidemiological study in Norway showed that risk factors related to DM did not explain the increased risk for UI among women with DM. This study revealed associations between DM management and complications and UI, but the biological and laboratory parameters do not appear to explain the previously documented association. However, associations were found between UI and some clinical correlates of DM [2].

DM has been established as an independent risk factor for UI, the involuntary leakage of urine [3]. DM causes debilitating and devastating complications not only during pregnancy but also after delivery [4]. Outside pregnancy, an association has been found between DM type 1

or 2 and a high prevalence of UI (30-60%) [5]. The risk conferred by DM appears to be in addition to other recognized risk factors for UI [6]. Current theories on the pathophysiology of lower urinary tract complications of DM include myopathic components of the pelvic floor [7, 8].

The role of pregnancy and childbirth in determining UI is still debatable. Many hypotheses attempt to explain the origin of UI during pregnancy, its association with vaginal delivery, and the protective role of cesarean section. The increased concentration of collagen and decrease in muscle fibers in the urethra of female rats after vaginal delivery may be one of the mechanisms in the development of UI in women [9]. However, Barbosa *et al.,* [10] showed that elective cesarean section was not sufficient to prevent UI two years postpartum.

UI is a debilitating disorder caused by malfunctioning of the urethral sphincter. Anatomical and histological properties of the sphincter, its innervations, and supporting structures are explained in relation to the closing mechanism of the bladder outlet [11]. Stronger clinical support for a causal relationship between decreased thickness of the urethral sphincter and UI has been provided [12].

Striated fibers are the dominating muscle component in the mid-urethra [13] and have been classified into two major groups: type I (slow-twitch) and type II (fast-twitch) based on the presence of myosin heavy

chain (MHC) isoforms. Slow-twitch type I muscle fibers are rich in mitochondria, present high oxidative capacity, and are resistant to fatigue. Conversely, fast-twitch type II muscle fibers have high glycolytic metabolism and fatigue easily [14]. The role of each fiber type in contraction of the striated sphincter is controversial and likely depends on the species studied and the method used for the determination of fast and slow types [15].

Given the high prevalence of UI among women with previous GDM, and considering that striated muscle is one of the two most important tissue types affected by insulin resistance and type 2 diabetes, the purpose of the present study was to evaluate the urethral striated muscle fiber composition in the urethra of the diabetic pregnant rat to better understand the influences of diabetes and pregnancy on the urethral muscle fibers. Alterations in the two basic types of urethral striated fibers: type I (slow) and type II (fast) in urethral muscle of pregnant diabetic rat were analyzed. We hypothesized that diabetes and pregnancy would detrimentally affect normal function of urethral striated muscle in rats, providing a model for further studies related to UI.

Materials and Methods

This study was developed in the Experimental Research Laboratory of the Department of Gynecology and Obstetrics, Botucatu

Medical School, UNESP-Univ Estadual Paulista, São Paulo, Brazil. Prior to the study, ethical approval was obtained from the Institutional Animal Care and Use Committee on Animal Experimentation of Botucatu Medical School-UNESP (Process number 668).

Six-week-old female and nine-week-old male Wistar rats, weighing approximately 180g and 220g, respectively, were allowed to adapt to the laboratory for seven days. The rats were kept in collective cages under controlled conditions of temperature (22 ± 3° C), light (12 hour light/dark cycles) and relative humidity (60 ± 5%). The animals were fed with laboratory chow (Purina®) and tap water *ad libitum* and cared for in accordance with the principles of the Guide for Care and Use of Experimental Animals.

The adult female rats were distributed among four groups:

Group 1: five virgin rats euthanized on the 28th day of the experiment;

Group 2: five pregnant rats that underwent cesarean section on day 21 of pregnancy and were euthanized immediately;

Group 3: five diabetic virgin rats euthanized on the 28th day of diabetes induction;

Group 4: five diabetic pregnant rats that underwent cesarean section on day 21 of pregnancy and were euthanized immediately.

Induction of Diabetes

Diabetes was induced by streptozotocin (STZ - SIGMA Chemical Company, St. Louis, MO, USA) seven days before mating. A dose of 40 mg/kg -body weight was administered by intravenous route to produce a permanent severe diabetic state. Blood glucose levels were measured at the beginning and end of the experimental period using glucose oxidase reagents strips (One-Touch Ultra Johnson & Johnson®, Milpitas, CA, USA). Only rats with glucose levels greater than 200 mg/dL were assigned to the diabetic groups [16].

Female rats (pregnant and diabetic pregnant groups) were mated overnight with non-diabetic male rats. The morning when sperm was found in the vaginal smear was designated gestational day 0.

On day 21 of pregnancy, fed rats were weighed in order to determine maternal weight gain (final weight - initial weight) and lethally anesthetized with sodium thiopental (Thiopentax® 3%). Following trichotomy of the abdominal region, the animal was placed in the dorsal decubitus position, and its ribs were fixed to the surgery table. Laparotomy was carried out by a midline incision beginning at the xiphoid cartilage and ending at the pubis. The intestinal loops were moved cranially for uterus exposure. Hysterectomy was carried out by sectioning of the ligament, artery and ovarian vein and incision of the

uterine body above the cervix. Incisions were then placed throughout the entire extension of the uterine horns, on their free margin and in the most avascular area. The fetus, amniotic sac and placenta were removed by slight traction (Figure 1). Following birth, offspring were weighed, anesthetized and euthanized.

The urethra and vagina were extracted as a unit to facilitate their handling (Figure 2). Each unit was immediately placed in a position suitable for transverse sectioning and was frozen with liquid nitrogen. Samples were stored at -80°C until sectioning and staining. Cryostat sections of 6-μm thickness were cut and stained with hematoxilin-eosin (H&E) to visualize nuclei, membranes, cytoplasm and connective tissue. Immunohistochemical procedures were performed on 6 μm thick serial cross-sections to visualize fast and slow myosin heavy chains (MHCfast and MHCslow). Antibodies WB-MHCf Novocastra (1:120) and WB-MHCs Novocastra (1:160) were used.

Data analysis

Study of the rat urethra was performed by morphological analysis, and a semi-quantitative method was used to analyze immunohistochemical staining of fast and slow type skeletal muscle fibers.

For this analysis, fast and slow type fibers were considered separately. The intensity of immunolocalization was evaluated by two independent readers and averaged. Striated muscle of the urethra was analyzed based on the following parameters: the presence of each type of fiber throughout circumference of the layer (++++ if layer complete throughout circumference and + if not complete), the thickness of muscle fiber layers (++++ for thickness of more than five layers and + for thickness of one muscle fiber layer) and the degree to which the layers maintained a normal anatomic localization (++++ normal anatomic localization, ++ intermediate anatomic localization, + loss of normal anatomic localization).

The scores for presence in circumference, thickness, and anatomic localization, based on the above criteria, were multiplied for each fiber type. The obtained values for fast fibers were then divided by those for slow fibers to establish the fast/slow index.

Convenient transformations (Neperian log) were performed in order to make offspring weight, maternal weight gain and glycemic data adjust to a symmetrical distribution and homogeneous variance. Analysis of Variance (ANOVA) was used followed by Tukey´s Multiple Comparison Test. Statistical significance was considered as $p<0.05$. Data were expressed as mean ± standard error of mean (SEM).

Results

Urethral histology

The transverse sections of the central part of the urethra in the virgin group showed different layers from the lumen to the periphery: stratified squamous epithelium (arrow), lamina propria (✸) spongy vascular plexus (P), smooth muscle: longitudinal (1) and circular (2) fibers and striated muscle (3) (Figure 3).

Morphological and semi-quantitative analysis of striated muscle fiber composition in the rat urethra

Virgin group

H&E-stained transverse cross-sections of the striated muscle fiber revealed many layers and compact outer circular layers. The fibers were long, with similar thickness throughout the circumference (Figure 4-A)**.**

Immunohistochemical staining revealed that the striated myofibers predominantly expressed the fast myosin heavy chain isoform. The layer containing fast fibers was thick and the fibers were present throughout the outer circular layer (++++) (Figure 4-B). The proportion of fast to slow fibers was 4:1 (Table 1). A thin, inner circular layer of slow striated muscle fibers was observed (+) with individual fibers being small and thin (Figure 4-C). The image suggested different localization patterns for

each type of fibers, with fast fibers being outermost and slow fibers innermost.

Pregnant group

H&E-stained transverse cross-section revealed that the appearance of the striated muscle layer was similar to that of the control group. An increase in connective tissue separated the fibers from one another. The most important finding in this group was the great interstitial spaces found between fibers (Figure 5-A).

Immunohistochemical staining revealed that the distribution of fast and slow fibers and the proportion between them were similar to those of the virgin group (4:1) (Figure 4-E-F) (Table 1).

Diabetic virgin group

H&E-stained transverse cross-section showed that the circular annulus was lost. There was obvious fiber thinning and atrophy, and the striated muscle was disrupted. There were few complete layers of striated muscle (Figure 5-B).

Immunohistochemical staining revealed that the specific localization for each type of fiber was lost, with co-localization of fast and slow fibers and a decrease in the proportion of fast to slow fibers for 1.5:1 (Figure H-I) (Table 1).

Diabetic pregnant group

H&E-stained transverse cross-section showed that the circular annulus was lost. The fiber layers were thin, atrophic, and disorganized, and the striated muscle was disrupted. The findings were similar to those of the pregnant group in relation to the increase in connective tissue separating the fibers from one another and the great interstitial spaces (Figure 5-C).

Immunohistochemical staining here also revealed a loss of specific localization for each type of fiber, with co-localization of fast and slow fibers and a decrease in the proportion of fast to slow fibers for 1.5:1 (Figure 6-B-C) (Table 1).

Maternal and perinatal results

Mean maternal weight gain and offspring weights from the pregnant group showed no significant statistically differences as compared to those from diabetic pregnant rats (Table 2).

The diabetic virgin and diabetic pregnant groups presented with increased glycemia during pregnancy as compared to virgin and pregnant groups ($p<0.05$) (Table 2).

Discussion

The goal of the present study was to gain a more comprehensive understanding of striated muscle fiber composition in the urethra of the pregnant diabetic rat and the proportion between two basic types of urethral striated muscle fibers: type I (slow-twitch) and type II (fast-twitch). It is of paramount importance not only to understand the effects of DM and pregnancy on striated muscle but also to develop new therapeutic strategies. Human studies are often limited due to ethical concerns, to the challenges of obtaining large tissue samples, and to the use of strictly-managed control groups. To better understand how different risk factors for UI affect the morphological properties of striated muscle, animal models are useful as the experiments are conducted under controlled conditions [17].

The striated muscle fiber composition of the urethra of the diabetic pregnant rat is discussed along with the importance of considering experimental conditions, and the possibility to include three control groups: virgin, pregnant and diabetic virgin. With this methodology, it was possible to separately analyze the influence of diabetes and pregnancy. Of particular note, we found that, relative to these three groups, the urethral striated muscles of diabetic pregnant rats present with the following: thinning and atrophy, disorganization, disruption of the

circular annulus associated with co-localization of fast and slow fibers, and a steady decrease in the proportion of fast to slow fibers (fast:slow 1.5:1). An increase in connective tissue separating the fibers from one another, as in the interstitial spaces between fibers, occurred as an effect of pregnancy on urethral muscle.

Thinning and atrophy, disorganization, and disruption of the circular annulus of striated muscle were extensive damages caused by diabetes [18, 19]. DM was related to accumulation of reactive oxygen species and tissue ischemia can interactively or independently contribute to the myopathy causes of skeletal muscle dysfunctions [20-21]. In our research laboratory, the relationship between oxidative stress and diabetes in pregnant rats was confirmed by Damasceno *et al.,* [22].

In analyzing this data, we were able to explain that extensive damage to striated muscle fibers characterized by reduced skeletal muscle mass and altered myofiber composition in diabetic pregnant rats links diabetes and pregnancy to UI. This specific loss of skeletal muscle mass is referred to as diabetic myopathy [23]. The present study confirms previous findings that diabetic myopathy and pregnancy are involved in the pathogenesis of urinary incontinence.

Differences in fiber type composition were detected in urethral striated muscle in diabetic pregnant rats in comparison to control groups. In the studied animals, the expression profile of fast to slow fibers

revealed two main differences. First, fast fibers lost their great predominance in relation to slow fibers. Second, the fast fibers lost their typical architecture, and the tissue was transformed into a mixture of slow and fast fibers. To the best of our knowledge, the findings are here described for the first time and may be labeled as a diabetic pregnant myopathy. Studies in animal models have shown a strong relationship between muscle fiber type and the development of diabetes [24].

Skeletal muscle is recognized not only for being responsible for movement but also for its function as the largest organ for glucose utilization. Our finding of increased type I slow-type fibers could be related to the abundant availability of lipids [25]. It is well established that changes in muscle fiber composition are often associated with glucose metabolism, diabetes and obesity [26]. Since muscle is a main site of glucose uptake, reduced muscle mass and changes in fiber type composition may directly impair acute glucose utilization. Skeletal muscle can adapt to functional and metabolic demands by remodeling with fiber-type switches to maintain a normal energy balance and utilization of nutrients.

Chen *et al.,* [14] confirmed a higher proportion of type I fibers and the presence of fast-to-slow fiber-type switching, which appears to be dissociated from the expected change in oxidative capacity. Our findings suggest that DM alters the profile of fast-to-slow fibers in the urethral

striated muscle of diabetic pregnant rats and that an eventual fiber-type switching could be present. The nature of the mechanism related to this altered fiber type in our model requires further investigation.

As the primary function of the lower urinary tract is the storage and expulsion of urine at the appropriate times, changes in the striated muscle composition could be related to the loss of type II fibers [27] or to the transformation of most type II fibers into type I fibers [28]. Given the limitations of this study, its results could represent muscle changes according to glucose levels.

The damages revealed by morphological studies demonstrate the impact of the association between diabetes and pregnancy on urethral striated muscle fibers, as three of the factors related to altered urethral striated muscle in diabetes and pregnancy – maternal weight gain, offspring weight and trauma related to vaginal delivery – were controlled. However, the results of our study should be interpreted with awareness of the following limitations: rats are quadrupeds; they have tails with associated musculature; and their bladders are abdominal rather than pelvic organs [18].

It is well-established that the functional capacity of a muscle is impaired when its fibers are injured [29]. As the function of skeletal muscle is determined by muscle mass and fiber composition [14], our exciting results provide evidence that diabetes and pregnancy injure striated

muscle and alter its fast and slow fiber composition. These data suggest that diabetic pregnant rats may present altered contractility of urethral striated muscle, supporting the high UI prevalence in women with previous GDM, two years after cesarean section [1].

The importance of this study is support of the previous hypothesis that diabetes and pregnancy detrimentally affect the normal function of urethral striated muscles in rats, providing a model for further studies.

Conclusion

This study allowed us to describe the morphological changes in muscle mass and in the fast-to-slow fiber profile in urethral striated muscle fibers of diabetic pregnant rats. The urethral striated muscles were found to be thin and atrophic, disorganized, and disrupted. They were associated with the loss of normal anatomic localization for each fiber type with co-localization of fast and slow and with the loss of predominance in the expression of fast fibers in relation to slow fibers. This suggests that UI in diabetic pregnant women may be attributed in part to the changes in urethral striated muscles.

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Competing interests

The authors declare that no competing interests exist.

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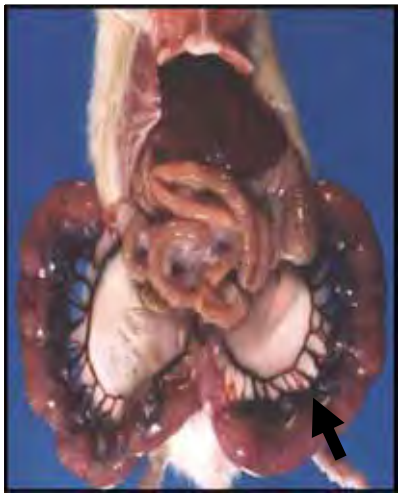

Figure 1. Photograph of anesthetized female rat showing the uterine horns. Arrow points the place where the horns were opened and the fetuses withdraw.

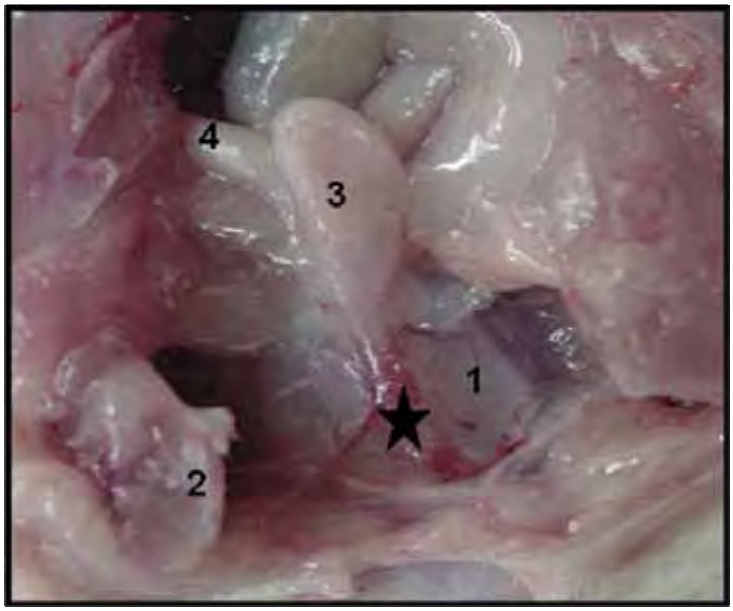

Figure 2. Photograph of pelvic region in female rat. Vagina (1), pubic symphysis(2), bladder (3), uterine horn (4), urethra (✶).

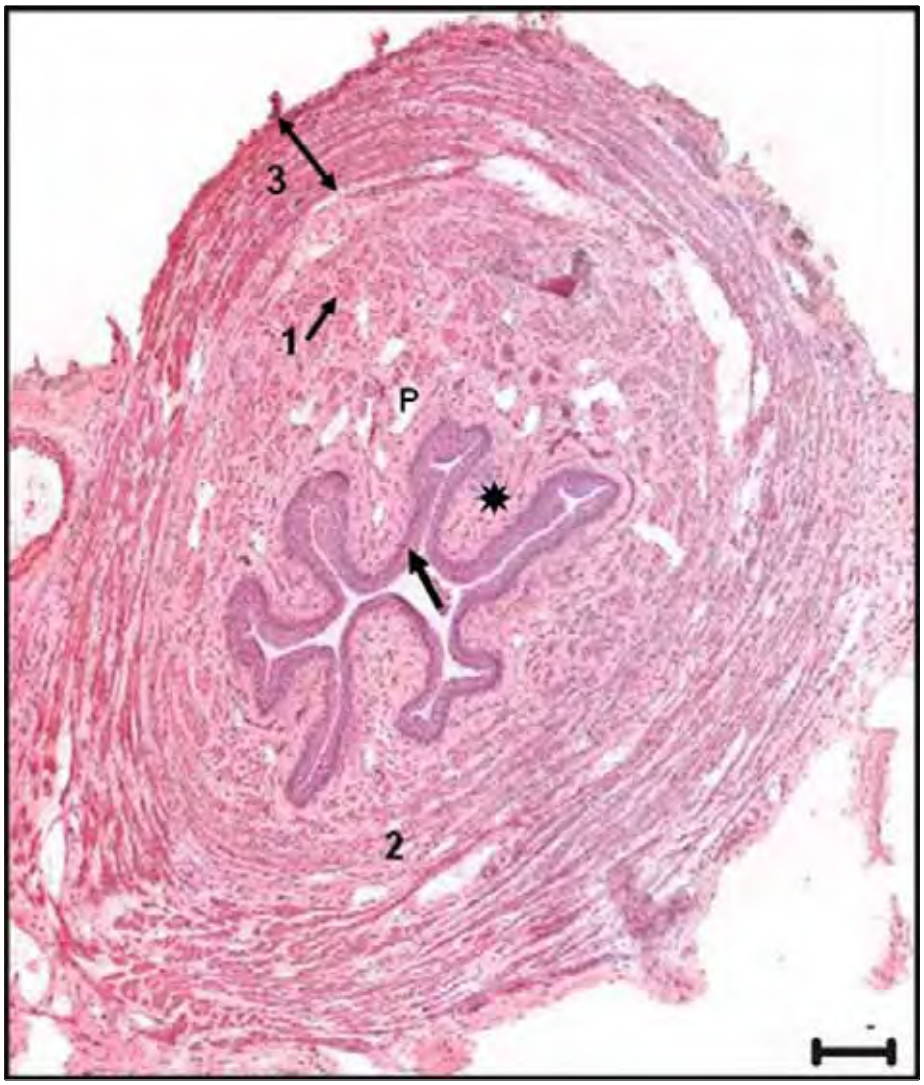

Figure 3. Microphotograph of transverse section of the central part of the urethra in a virgin female rat. Different layers are evident from the lumen to the periphery: epithelium (arrow), lamina propria (✸), spongy vascular plexus (P), smooth muscle: longitudinal (1) and circular (2) oriented fibers, and striated muscle (3). H&E stained. Scale bar = 100 µm.

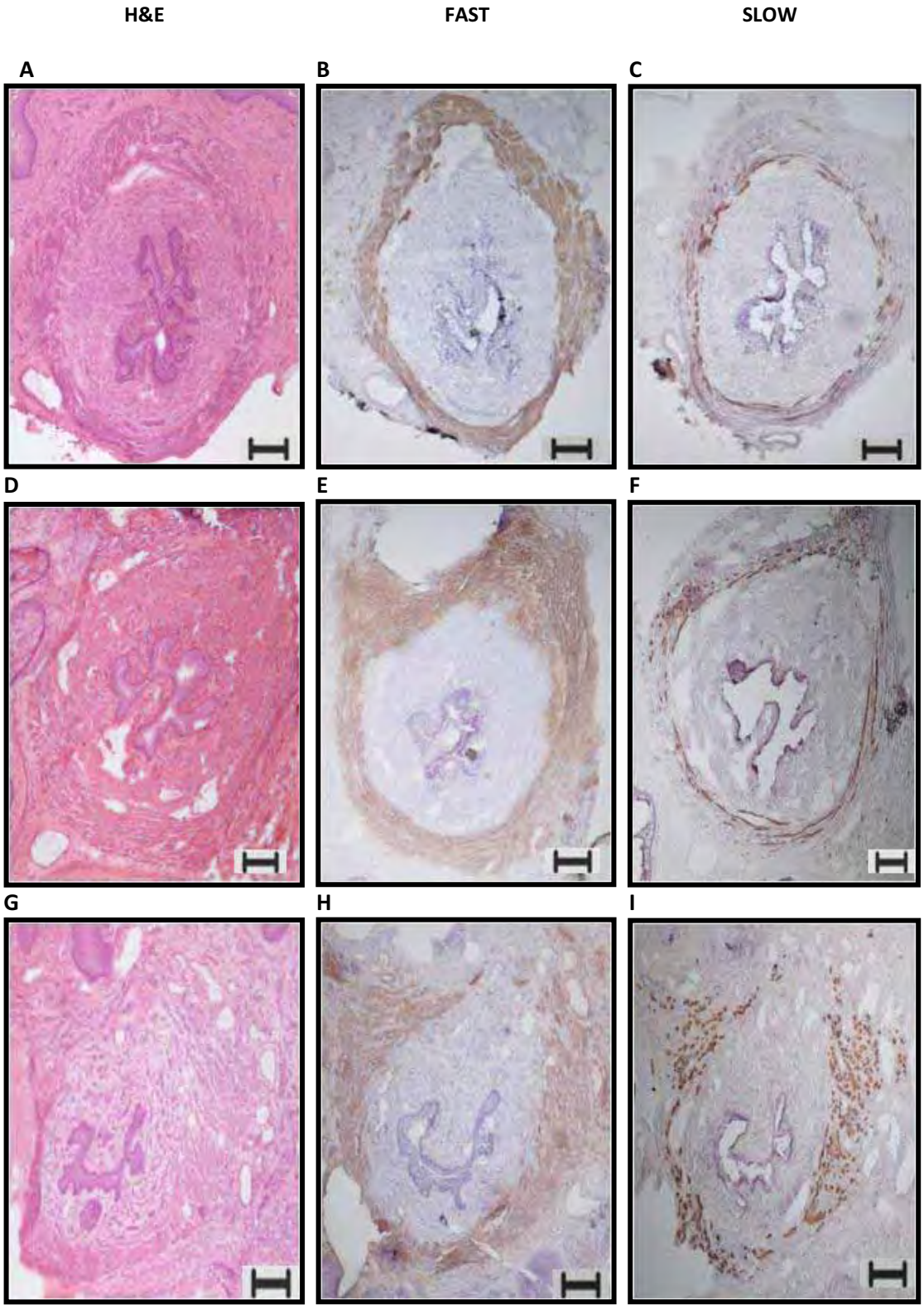

Figure 4. Microphotographs of transverse section of the urethra in virgin group (A, B, C), pregnant group (D, E, F), diabetic virgin group (G, H, I). H&E stained (H&E); immunohistochemical staining to visualize fast (FAST) and slow (SLOW) myosin heavy chain (MHCf, MHCs) in the striated muscle fibers. Scale bar = 100 μm

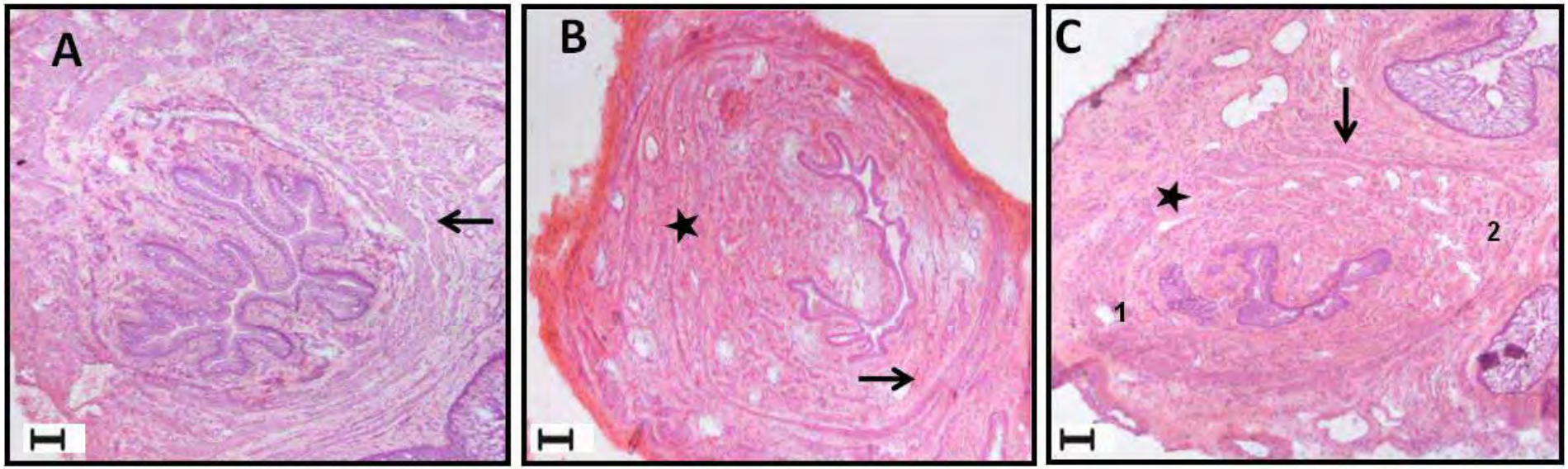

Figure 5. Microphotographs of transverse section of the rat urethra: A - pregnant group: increase in connective tissue separated the fibers from one another (arrow). B - diabetic virgin group: the striated muscle showed thinning and atrophy (arrow), and disruption (★) C- diabetic pregnant group: the striated muscle showed intersticial spaces (1), thinning and atrophy (2), disorganization (arrow), and disruption (★). H&E stained. Scale bar = 100 µm.

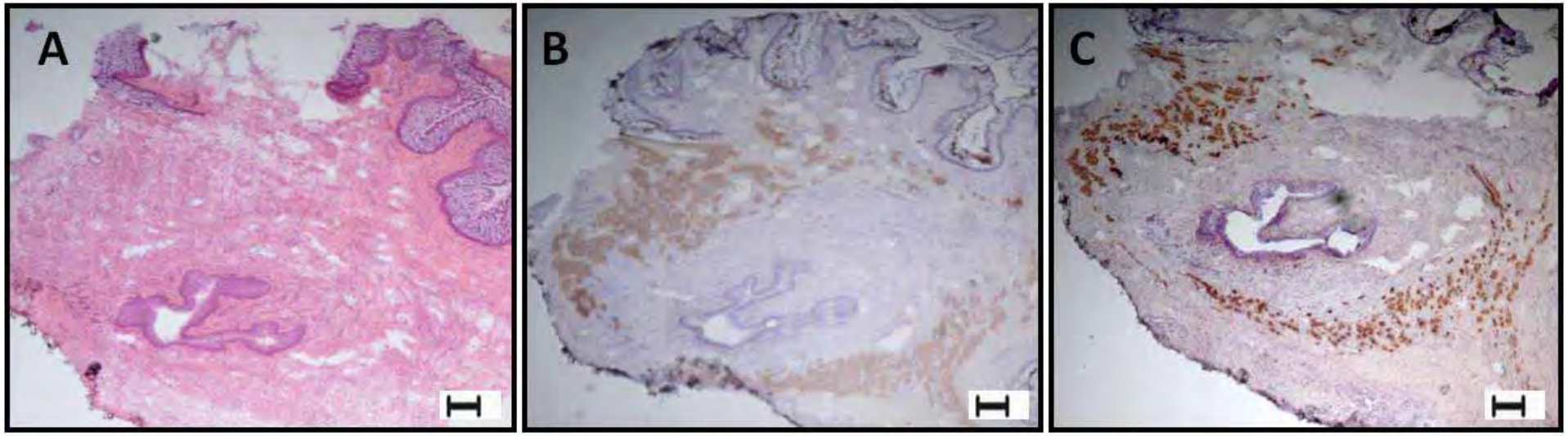

Figure 6. Microphotographs of transverse section of the rat urethra in diabetic pregnant group. H&E stained (A), immunohistochemical reaction for fast (B) and slow (C) myosin heavy chain (MHCf, MHCs). Scale bar = 100 µm

Table 1. Semi-quantitative analysis of slow and fast fibers according the presence of each type of fiber throughout circumference of the layer; thickness of the muscle fiber layer; the degree to which the layers maintained a normal anatomic localization; fast index; slow index; and fast:slow index in each group.

Groups	**Virgin**	**Pregnant**	**Diabetic Virgin**	**Diabetic pregnant**
Fast Throughout circumference/thickness/ normal anatomic localization	++++/++++/++++	++++/++++/++	+++/++++/+	+++/+++/+
Slow Throughout circumference/thickness/ normal anatomic localization	++++/+/++++	++++/+/++	+++/+++/+	+++/++/+
Fast index	64	32	12	9
Slow index	16	8	9	6
Fast: Slow index	4:1	4:1	1.5:1	1.5:1

Table 2. Maternal weight gain (g) and offspring weight (g) in pregnant and diabetic pregnant groups. Maternal glycemia (mg/dL) from virgin, pregnant, diabetic virgin and diabetic pregnant groups at beginning and end of the experimental period.

Groups	**Virgin**	**Pregnant**	**Diabetic Virgin**	**Diabetic pregnant**
Maternal weight gain (g)		115.4±16.3		72.2 ± 21.5
Offspring weight (g)		82.6 ± 10.6		67.2 ± 14.3
Maternal glycemia (mg/dL) beginning of experiment	81.0 ± 4.4	112.8 ± 4.0	568.4 ± 38.0*	544.0 ± 36.8*
Maternal glycemia (mg/dL) end of experiment	81.6 ± 5.0	82.6 ± 7.7	584.8 ± 33.9*	497.8 ± 60.4*

Values are reported as mean ± SEM.
*$p<0.05$ – significant statistically difference compared to virgin and pregnant groups (Tukey's Multiple Comparison Test).

Vi, então, um novo céu e uma nova terra! “Eis aqui o tabernáculo de Deus com os homens. Habitará com eles e serão seu povo, e Deus mesmo estará com eles. Enxugará toda lágrima de seus olhos e já não haverá morte, nem luto, nem grito, nem dor, porque passou a primeira condição”. “Eis que faço nova todas as coisas.”

Apocalipse 21:1

Capítulo 3

Alterações morfológicas das fibras tipos I e II do músculo estriado uretral de ratas prenhes diabéticas

Autora: Gabriela Marini

Orientadora: Marilza Vieira Cunha Rudge

Co-orientadoras: Angélica Mércia Pascon Barbosa, Selma Maria Michelin Matheus

Banca: Marilza Vieira Cunha Rudge, Débora Cristina Damasceno, Manoel João Batista Castello Girão

Data da apresentação: 25 de fevereiro de 2010

Este capítulo foi redigido de acordo com as normas de publicação de resumos de Dissertação da **Revista Brasileira de Ginecologia e Obstetrícia**, para a qual será submetido.

RESUMO

Objetivos: avaliar as alterações morfológicas das fibras musculares estriadas tipos I e II da uretra de ratas prenhes diabéticas submetidas à cesárea. **Métodos:** Foram avaliadas 20 ratas Wistar distribuídas em quatro grupos: virgem, prenhe, diabético virgem e prenhe diabético. Os três primeiros grupos foram estudados para servir como controle do grupo principal, o prenhe diabético. O diabete foi induzido com streptozotocin na dose de 40mg/kg de peso corpóreo. O critério de inclusão foi uma glicemia acima de 200mg/dL. No final do experimento, as ratas foram anestesiadas e eutanasiadas para realização da laparotomia exploratória. A vagina e a uretra foram retiradas em monobloco, congeladas em nitrogênio líquido e mantidas a -80°C. O bloco foi submetido a cortes em criostato (6 µm de espessura). As lâminas foram coradas por H&E e utilizados anticorpos anti-miosina lenta e rápida para tipagem das fibras. Foi realizada análise morfológica e semi-quantitativa dos quatro grupos. **Resultados:** O músculo estriado uretral do grupo prenhe diabético apresentou: adelgaçamento, atrofia, desorganização e rompimento associado à perda de localização anatômica normal das fibras rápidas e lentas e diminuição na proporção de fibras rápidas. **Conclusões:** Este estudo sugere que o binômio diabete e prenhez danificou o músculo estriado uretral e alterou a composição e a distribuição das fibras tipo I e II .

Palavras-chave: diabete, fibra muscular estriada, prenhez, uretra

Anexos

ANEXO 1 – Aprovação do Comitê de Ética

unesp **Universidade Estadual Paulista**
Faculdade de Medicina de Botucatu

Distrito Rubião Junior, s/nº - Botucatu – S.P.
CEP: 18.618-970
Fone/Fax: (0xx14) 3811-6143
e-mail secretaria_capellup@fmb.unesp.br
Instituída na Faculdade de Medicina através da Portaria do Diretor nº 30 de 26/04/99

Comissão de Ética em Experimentação Animal

Botucatu, 28 de maio de 2.009 OF. 007/2009-CEEA

Ilustríssima Senhora
Profª Drª Marilza Vieira Cunha Rudge
Departamento de Ginecologia e Obstetrícia da
Faculdade de Medicina de Botucatu.

Cara Drª Marilza,

Em atenção à sua solicitação contida no ofício datado de 08 de maio de 2.009, informo que em reunião da CEEA de 28 de maio de 2.009, foi autorizada a inclusão dos sub-grupos **"estudo de ratas nulíparas diabéticas e prenhes diabéticas"**, referentes ao Protocolo **668-2009**, aprovado por esta Comissão em 27/03/2008.

Com a inclusão dos "sub-grupos", o Protocolo 668-2009, teve seu título alterado na seguinte conformidade:

Título anterior: "Estudo morfológico da musculatura vaginal e uretral em ratas prenhes: montagem de modelo experimental" - autoria de Gabriela Marini, orientada pela Profª Drª Marilza Vieira Cunha Rudge.

Título atual: "Estudo morfológico da musculatura vaginal e uretral em ratas prenhes diabéticas: montagem de um modelo experimental" - autoria de Gabriela Marini, orientada pela Profª Drª Marilza Vieira Cunha Rudge.

Atenciosamente,

Profª Drª Regina Helena Garcia Martins
Presidente da CEEA.

ANEXO 2 – Alteração no título

unesp

DIVISÃO TÉCNICA ACADÊMICA

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JUSTIFICATIVA DE ALTERAÇÃO NO TÍTULO DO PROJETO DE PESQUISA

Declaramos que o Projeto de Pesquisa "Estudo morfológico da musculatura vaginal e uretral em ratas prenhes diabéticas: montagem de um modelo experimental" aprovado pelo CEP em 27/ 03/ 2008, teve seu título alterado para ***"Alterações morfológicas das fibras tipos I e II do músculo estriado uretral de ratas prenhes diabéticas"****, sem nenhuma alteração no seu conteúdo metodológico da época de apresentação para análise do CEP.*

A presente alteração foi efetuada somente para adequação do título da Dissertação de Mestrado.

Botucatu, 27 de janeiro de 2010

Nome/Assinatura da aluna Gabriela Marini Gabriela Marini

Nome/Assinatura da orientadora Marilza Vieira Cunha Rudge Rudge

Programa de Pós Graduação em Ginecologia e Obstetricia

11:15 27/01/2010 000000 COMITE DE ETICA EM PESQUISA FMB - UNESP

✓ **Preencher formulário em 2 vias e protocolar no respectivo CEP**

ANEXO 3 – Ofício do envio para publicação do Capítulo 1

Article title: Diabetes in pregnancy and urinary incontinence: a little acknowledged association

MS ID : 1906283565344512

Authors : Gabriela Marini, Angelica MP Barbosa, Débora C Damasceno, Rodrigo A Castro, Selma MM Matheus and Marilza VC Rudge
Journal : Diabetology & Metabolic Syndrome

Dear Dr Marini

Thank you for submitting your article. This acknowledgement and any queries below are for the contact author. This e-mail has also been copied to each author on the paper, as well as the person submitting. Please bear in mind that all queries regarding the paper should be made through the contact author.

Regards

The Diabetology & Metabolic Syndrome Editorial Team

e-mail: editorial@dmsjournal.com
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Terça-feira, 12 de Janeiro de 2010 12:55