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Análise morfológica do músculo reto abdominal de ratas prenhes diabéticas

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestre em Ginecologia, Obstetrícia e Mastologia

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Epígrafe

“De baixo do céu há momento para tudo, e tempo certo para cada coisa: Tempo para nascer e tempo para morrer. Tempo para plantar e tempo para arrancar a planta. Tempo para matar e tempo para curar. Tempo para destruir e tempo para construir. Tempo para chorar chorar e tempo para rir. Tempo para gemer e tempo para bailar. Tempo para atirar pedras e tempo para recolher pedras. Tempo para abraçar e tempo para para se separar. Tempo para procurar e tempo para perder. Tempo para guardar e tempo para jogar fora. Tempo para rasgar e tempo para costurar. Tempo para calar e tempo para falar. Tempo para amar e tempo para odiar. Tempo para guerra e tempo para a paz.”

Eclesiastes 3

Sumário

Estado da arte da Linha de pesquisa: "Incontinência Urinária e diabete na gestação"

A elevada ocorrência de incontinência urinária (IU) e disfunção muscular do assoalho pélvico (DMAP) em portadoras de Diabete mellitus gestacional (DMG) evidenciada por Barbosa em sua tese de doutorado em 2009 junto ao Programa de Pós-graduação em Ginecologia, Obstetrícia e Mastologia da Faculdade de Medicina de Botucatu-UNESP, publicada em 2011 foi o inicio da Linha de pesquisa:" Incontinência urinária e diabete na gestação". A prevalência elevada de IU gestacional em diabéticas foi de 50,8% *vs*. 31,6% nas normoglicêmicas e dois anos pós-parto cesárea a IU nas diabéticas acometeu 44,8% *vs.* 18.4% nas normoglicêmicas. Essa diferença foi significativamente mais elevada nas mulheres com DMG prévio do que entre as normoglicêmicas. A análise multivariada demonstrou que o DMG foi fator de risco independente para a ocorrência de IU gestacional (OR 2,26; IC 95%: 1,116-4,579). Também foi demonstrado que as gestantes com DMG prévio dois anos após parto cesárea têm disfunção muscular do assoalho pélvico (DMAP) (1). As associações entre hiperglicemia e DMAP e entre gestação e DMAP estão bem estabelecidas, entretanto a associação entre hiperglicemia na gestação, DMAP e IU é escassa na literatura.

A partir desse resultado clínico surpreendente decidimos pelo rumo da pesquisa translacional procurando as explicações para esse achado nos músculos envolvidos no mecanismo de continência urinária em ratas prenhes com diabete moderado e grave. Os resultados dessas investigações evidenciaram alterações musculares estruturais e ultraestruturais caracterizados por: adelgaçamento, atrofia, aumento de colágeno na área de músculo estriado, aumento de vasos, acúmulo de mitocôndrias, além de gotas de lipídios e grânulos de glicogênio, desorganização e rompimento de fibras, associado à perda da

localização anatômica normal das fibras rápidas e lentas (tipos I e II) e diminuição na proporção de fibras rápidas na uretra (2,3). Esses resultados experimentais estão sendo analisados em conjunto com a *Case Western Reserve University (CWRU) em Cleveland-Ohio-USA* numa parceria internacional importante nas pesquisas e nas análises e discussões conjuntas com o grupo dos Prof. Firouz Daneshgari e Adonis Hijaz.

Com essas pesquisas experimentais realizadas e publicadas evidencia-se lesão importante dos músculos do assoalho pélvico de ratas prenhes e diabéticas que suportam a hipótese fisiopatológica da lesão muscular como fator fundamental na IU em diabéticas.

Em continuidade a esse conhecimento fisiopatológico da disfunção muscular do assoalho pélvico, deparamos com a dificuldade ética de obter biópsia de músculos do assoalho pélvico em gestantes diabéticas para confirmar achados similares. A análise da literatura evidenciou que os músculos abdominais possuem grande participação nos mecanismos de continência urinária. Estudos recentes têm focado a relação entre os músculos do assoalho pélvico e os músculos abdominais e mostram que a co-contração dos músculos do assoalho pélvico e dos músculos abdominais ocorre tanto durante a contração voluntária do assoalho pélvico como durante manobras abdominais.

A co-contração dos músculos abdominais parece contribuir para gerar força para a contração do AP. Alguns pesquisadores começaram a investigar essas relações em mais detalhe. Sapsford *et al.* (4) descobriram que os músculos do AP foram ativados durante as contrações musculares abdominais e que o inverso também é verdadeiro, isto é, os músculos abdominais foram ativados durante as contrações do AP. Da mesma forma, Neumann e Gill (5) relataram que não foi possível para mulheres continentes contrair totalmente o AP sem contrair os músculos abdominais. Bo *et al* (6), ao estudarem três fisioterapeutas que estavam bem treinadas para realizar as contrações do AP de forma isolada, observaram que estas profissionais não foram capazes de realizar a máxima contração do AP sem aumento

na atividade da eletromiografia na porção inferior do músculo reto abdominal. Em conjunto, estes estudos indicam que há co-contração entre os músculos abdominais e do AP. Entretanto há grande dúvida se essa atividade conjunta dos músculos do assoalho pélvico e reto abdominal na continência urinária está associada com alterações morfológicas similares.

Esta dissertação de mestrado foi desenvolvida nesta etapa do conhecimento que vem sendo desenvolvido nesta linha de pesquisa e também considerando os conhecimentos atuais de verdadeiro "cross- talking" entre os músculos estriados do assoalho pélvico e os músculos da parede abdominal (7).

Para a análise dos nossos dados recebemos Bolsa BEPE-Fapesp (processo número: 14144-7) para a Case Western Reserve University- Cleveland- USA com a supervisão do Prof. Adonis Hijaz. Ao longo do período de desenvolvimento do projeto, tivemos a oportunidade de apresentar três trabalhos relativos à dissertação em congressos nacionais e internacionais (ANEXOS).

Esta dissertação está dividida em 2 capítulos formatados de acordo com as normas das revistas a que serão submetidos e denominados:

Capítulo 1: Skeletal muscle wasting and fast/slow fiber type profile changes caused by diabetes in pregnant rats.

Capítulo 2: Cross talk between abdominal muscle and pelvic floor muscle in urinary continence mechanisms

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ANEXOS

ICS 2014
Rio de Janeiro

CERTIFICATE of PRESENTATION

This is to certify that

G Vesentini, F Marini, F Piculo, S Matheus, AP Almeida, D Damasceno, A Barbosa & M Rudge

Participated at

The 44th Annual Meeting of the International Continence Society (ICS)
23rd October, 2014, Rio de Janerio, Brazil

by presenting the following open discussion poster

INFLUENCE OF DIABETES AND PREGNANCY IN ULTRASTRUCTURAL ANALYSIS OF THE RECTUS ABDOMINIS MUSCLE AND EXTRACELLULAR MATRIX IN RATS

Carlos D'Ancona.
Annual Meeting Chair.
ICS 2014 Rio de Janeiro

Nucelio Lemos.
Scientific Chair.
ICS 2014 Rio de Janeiro

ICS 2014
Rio de Janeiro

Delegates Choice Award for Open Discussion Posters:

Anatomy & Biomechanics

80 - Co-contraction of the abdominal muscles and pelvic floor muscles in urinary continence

Vesentini G, Marini G, Piculo F, Almeida A P, Damasceno D, Barbosa A, Rudge M

Carlos D'Ancona.
Annual Meeting Chair.
ICS 2014 Rio de Janeiro

Nucelio Lemos.
Scientific Chair.
ICS 2014 Rio de Janeiro

COGI

Certificate of Attendance

This is to certify that

Giovana Vesentini

Presenter of the Poster Presentationn

RECTUS ABDOMINIS MUSCLE ATROPHY INDUCED BY DIABETES IN PREGNANT RATS

has attended

The 20th World Congress on
Controversies in Obstetrics, Gynecology & Infertility (COGI)

Paris, France, December 4-7, 2014

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Capítulo 1

CROSS-TALK BETWEEN ABDOMINAL MUSCLES AND PELVIC FLOOR MUSCLES IN URINARY CONTINENCE MECHANISMS

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Este capítulo foi redigido de acordo com as normas de publicação da revista **Neurology and Urodynamics**, para a qual será submetido.

ABSTRACT

Aims: The Pelvic Floor Muscle training is a well known treatment for urinary incontinence. Researches has been shown a cross- talk between the abdominal muscle (AM) and PFM. The aim of this study is to perform a literature review of the papers published in recent years that relates PFM contraction associated with AM contraction. **Methods:** Literature review of articles in the last 14 years on the database PubMed. **Results:** A very few articles was founded, one review and the other studies investigated this cross-talk in continent and/or incontinent woman. In all of these studies there is a strong evidence of both muscular groups co-activation. **Conclusions:** Besides the scarce studies, the cross talk was described in all of them. Maybe the combined association between them can improve a new treatment for UI.

Key words: Pelvic floor muscle, abdominal muscle, biomechanics, urinary incontinence.

INTRODUCTION

The costs related to Urinary Incontinence (UI) are estimated in annual spending of 12 billion dollars and increase every year (1). It is a significant problem among women with estimated prevalence of 25% to 45% throughout the world (2). It is a common symptom affectting woman in all ages with severe impact on their lives and considerable resource implications for the health service (3). Stress urinary incontinence is defined as involuntary urine loss on effort or exertion or on sneezing or coughing (3). Kegel was the first to report effect of pelvic floor muscle training (PFM) to treat UI (4). Strengthening of pelvic floor muscles (PFM) is the first line of management for treatment option of UI and the effectiveness of this treatment has been proving in numerous randomized controlled trials and reviews (5-8).

Researches, has been showing new strategies involving a model of abdominal muscles (AM) training to stimulate tonic PFM activity. This approach is based on the understanding that synergistic co-activity of the PFM and AM that occurs in normal activities. This new method has been suggested to improve clinical outcomes of PFM dysfunction (9, 10), since UI might be associated with a changed in activation of abdominal muscles, chest wall and diaphragm which affects the recruitment pattern and temporal regulation of PFM during daily activities (10-13).

The abdomino-pelvic muscles form a cylinder, which respond rapidly to tasks such as intra-abdominal pressure (IAP), trunk muscle activity, posture and to the varied continence and respiratory demands (12-14).

Abdominal muscles and PFM: anatomy and biomechanics

The best predictor of muscle function is its skeletal muscle architecture. The organization of muscle fiber-type relative to the axis predicts the muscle force and moving

capacity, determined by its active range of contraction (excursion) and how fast it contracts (velocity) (15,16). Thus, the understanding of the muscle architecture of AM and PFM can add new information about the synergism between them.

Abdominal Muscles Anatomy and Function

The abdomen is a compartment extending from the bottom of the chest to the top of the pelvis and lower limbs. Provides shelter to the principal organs of digestive system, parts of urinary system. The main part it is compound for skeletal striated muscle, laterally of the abdominal wall, there is three layers of muscle, transversus abdominis (TrA), obliquus internus abdominis (OI), obliquus externus abdominis (OE) and anteriorly the rectus abdominis (RA). A coordinated action these muscles surrounding this cylinder generate and control intra abdominal pressure (IAP), other trunk muscles can modulates this pressure (17,18). The abdominal muscles is important in controlling posture, stability, and trunk movement and is an important element in the performance of movements of the extremities (19,20).

Pelvic Floor Muscles Anatomy and Function

The descriptor “pelvic floor” (PF) is used to refer all the structures in pelvic cavity (21), major structures involved in proper pelvic floor functioning as vagina, pelvic floor skeletal muscles, and innervation (22). The static support is provided by the bony pelvis integrated with additional anatomical systems as the musculoskeletal, the fascial, and the visceral systems. PF support the base of the abdominal cavity and connect inferiorly through the bladder, urethra, uterus, and rectum, the endopelvic fascia, the deep PFMs, and the perineal (9,23). The deep PFM compound by levator ani (pubococcygeus, puborectalis, and iliococcygeus muscles) and coccygeus (23,24).

Physiological mechanisms of urinary continence

The PFM provide support to the viscera, constricting urethra, vagina, and anal canal, and at the same time allowing movement of contents from one cavity to another (urination, defecation, parturition) (24). Also, surround the pelvic opening, during a voluntary contraction, elevate the pelvic floor, working in synergism with urethra and increase the intra-urethral pressure, keep the pelvic organs inside the pelvis, stabilize and prevent leakage during rise in intra-abdominal pressure (13,25,26). During the incorrect perform, the PF depress and may actually increase the stress on the ligaments and fascia and worsen symptoms of prolapse and incontinence (27).

The term cross talk is frequently described in EMG studies, when besides the signal of surface electrodes determinate the level of specific muscle activation, record signals generated by muscles other than the muscle of interest (28). Understanding the biomechanics of PFM and AM it is possible to detect a physiological cross-talk important to promote urinary continence. Incorporating new therapeutic strategies may be beneficial in getting better results and lower health costs. Therefore, the aim of this study is to perform a literature review of the papers published in recent years that relates pelvic floor muscle contraction associated with AM contraction in women with and/or without incontinence.

METHODS

A literature review of articles in the last 14 years on the database PubMed using the following terms: "Urinary Continence", "Abdominal Muscles", "Pelvic Floor Muscles", "Co-activation", "Exercise" and "Woman" was performed. Considering the cross-talk between AM and PFM the criteria to include in this study was the co-contraction of the PFM during different AM contractions.

RESULTS

Figure I shows the studies found in the review. It was found 162 articles. Of these, 74,07% (120) were related to isolated contraction of the PFM, 6,17% (10) not related to the conservator treatment of UI, 15,43% (25) related to other themes, and 4,33% (7) to the associated contraction of AM and PFM. Considering these 4,33% (7) as total articles studied, among these, 42,86% (3) was a comparison with continent and incontinent women, 42,86% (2) only continent women and 14,28% (1) a literature review. Regarding the publication year, 14,28%% (1) of articles found are considered current, with subsequent publication to 2010 and 85,72% (6) in the year preceding 2010.

DISCUSSION

The cross-talk is shown in some studies and appears to overflow of activity into the PFM muscle. As AM is involved in the urinary continence mechanisms, several research groups focused on understanding the relationship between the muscles of the pelvic floor and abdominal muscles, and found that co-contraction of the pelvic floor muscles and abdominal muscles occurs both during voluntary contraction of the pelvic floor as during abdominal operations (10,12).

Sapsford et al (29) recorded the electromyography (EMG) of transversus abdominis (TrA), obliquus internus abdominis (OI), obliquus externus abdominis (OE), and rectus abdominis (RA) in seven participants with no symptoms of PFM dysfunction, and provides evidence that increased activity of the abdominal muscles with maximal activation of the PFM. The results of this study indicate that abdominal muscle activity is a normal auxiliary to contraction of the PFM and should not be discouraged. If abdominal muscle activity is discouraged during PF exercise, this may limit the optimal PFM response. These findings indicate that PF muscles may be used to initiate contraction of the abdominal muscles and

abdominal muscle activity should be expected (and not avoided) during the performance of exercises for PF muscles (29).

Later Smith et al. (30,31) studied the different activity of the abdominal and PF muscles in continent and incontinent women during a sudden and expected perturbation of the trunk, the different postural response of the PF muscles. Sixteen women with stress urinary incontinence and fourteen women with no history of incontinence were evaluated. The addition of load suggests that PF EMG and trunk muscles increased in response to the postural challenge when the postural perturbation was unexpected, EMG responses were greater than in trials when it was predictable. Women with incontinence had greater PF muscle activity compared to continent women before and after the postural perturbation despites women with incontinence may have reduced muscle mass and maximal ability, they may use greater PF muscle activity during postural perturbations and lumbopelvic instability (30,31).

Spasford et al.(32), analyze continent and incontinent women during activity of the PFM and AM varied in three different sitting postures in parous women with and without stress urinary incontinence. The PFM was affected by sitting posture in women with and without SUI, although the incontinent women, had less PFM EMG activity and a tendency toward greater activity of abdominal muscles than the continent women, activity of the PFM and abdominal muscles increased in both groups when moving moviments, also the lower activity of the PFM was associated with reduced lumbar lordosis (32).

Urethral pressure increases during voluntary pelvic floor (PF) muscle contractions in healthy women. Madill and McLean (33) investigated how these muscles work together to generate the rises in urethral pressure that are necessary to overcome rises in intra-abdominal pressure and how these muscles might work together to maintain continence. Fifteen healthy continent women participated. They performed three maximal contractions

of the abdominal muscles and of their PFM, the muscle activity was recording with EMG. They founded that during voluntary PFM contractions, an increase in lower intravaginal pressure is not solely the product of PFM activation, but involves a specific co-ordination between the PFM, TrA, RA, and IO muscles. The abdominal muscles in continent women contribute significantly to the development of lower intravaginal pressure during voluntary PFM contractions, particularly in the latter part of the contraction (33). Later, Sapsford et al. (34) investigated whether specific abdominal muscle actions change the urethral pressure, evaluating seven healthy woman during abdominal and PFM contraction. This study demonstrates that urethral pressure can increases during tasks with gradual increases in abdominal muscle activity and IAP and that synergistic activity of the abdominal and PFM can contribute to urethral closure in healthy woman (34). This new knowledge is very important and must be considered in the treatment of women with UI.

CONCLUSIONS

There is a strong evidence of AM participation in the urinary continence mechanisms, mainly the TrA, OI and RA confirming the cross-talk between them. Understanding in details the urinary continence mechanisms is the main goal to avoid the UI. The PFM training is a well-established training for a UI, but the AM training combine may be assist in the optimal level of the PFM muscle recruitment and which that a new tool for the treatment.

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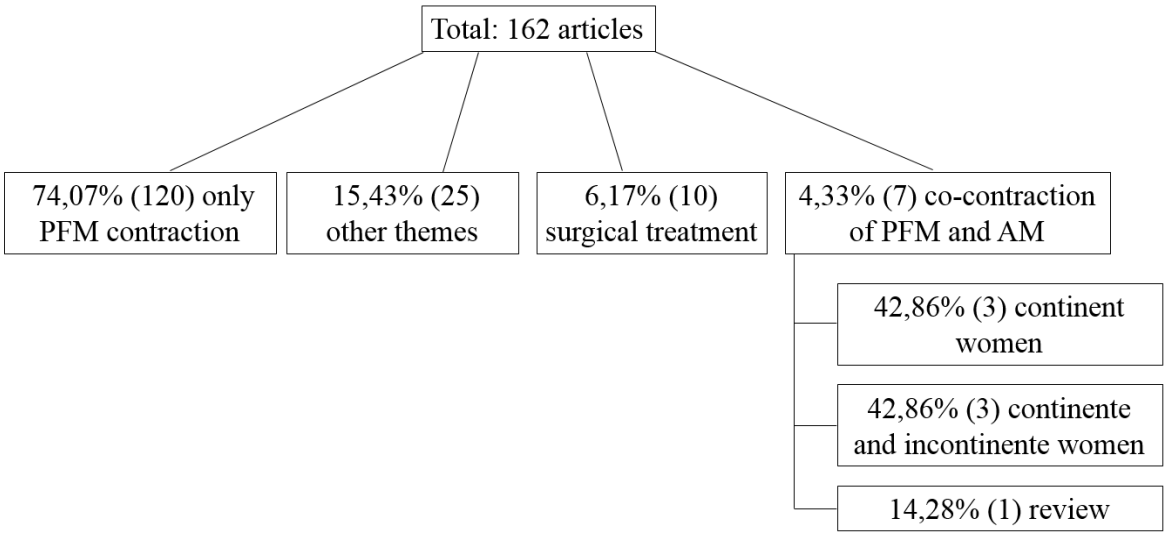

Figure 1. Studies founded on co-Contraction of the pelvic floor muscles during abdominal muscle contraction.

Table 1. Studies on Co-Contraction of the Pelvic Floor Muscles (PFM) During Abdominal Muscle Contraction.

Authors	Year	N	Population	Method for measurement	Muscle activity tested	Results
Sapsford *et al*	2001	7	Continent	EMG	Contraction of PFM in three lumbar positions	Increase activity of the AM during contractions of the PFM in at least one of the spinal positions
Madill and McLean	2006	15	Continent	EMG and Modified Femiscan™ probe	Record of the PFM activity during contractions of RA, TA, IO and EO.	Contraction between the PFM, TA, RA and IO muscle increase lower intravaginal pressure.
Smith *et al*	2007	16 w/SUI and 14 continent	Continent and Incontinent	EMG	Record of the PFM and AM activity during postural pertubation	Incontinent women have PFM and AM increased muscle activity during postural perturbations
Smith *et al*	2007	16 w/SUI and 14 continent	Continent and Incontinent	EMG	Record PFM and AM activity during rapid arm movements.	In incontinent women PF EMG decreased before the postural activation and increase delayed postural PF activity
Sapsford *et al*	2008	8 w/SUI and 9 continent	Continent and Incontinent	EMG	Contraction of PF muscles in three sitting postures	The PFM is affected by sitting posture in women with or without incontinence, but the symptomatic woman had less PFM activity and a tendency toward greater activity of superficial AM.
Sapsford *et al*	2013	7	Continent	Pressure sensors	Lower abdominal in-drawing, abdominal bulgin and PFM contraction with bladder empty and filled.	Urethral pressure increased during PF muscle contractions and abdominal in-drawing and did not differ between bladder status.

SUI: Stress Urinary Incontinence; EMG: Electromyograph; PFM: Pelvic Floor Muscle; AM: Abdominal Muscle; RA: Rectus Abdominis; TA: Transversus Abdominis; IO: Internal Obliques; EO: External Obliques.

Capítulo 2

SKELETAL MUSCLE WASTING AND FAST/SLOW FIBER TYPE PROFILE CHANGES CAUSED BY DIABETES IN PREGNANT RATS

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ABSTRACT

Objectives: To analyzed in an integrated way the effects of DM in different intensities associated with pregnancy in the rectus abdominis muscle of rats and compare with previous results of urethral striated muscle. **Methods:** Two hyperglycemic levels was studied the long-term mild diabetes, when female newborns received *streptozotocin* (100 mg/kg body weight, sc route) and short-term severe diabetes (blood glucose level >300 mg/d) the adult animals received *streptozotocin* at 50 mg/kg, ip route. At day 21 of pregnancy the rats were euthanatized and the lower-third rectus abdominis muscle were harvested and analyzed by picrosirius red staining for collagen and muscle components, immunohistochemistry for fast and slow muscle fibers and transmission electron microscopy for ultrastructural analysis. **Results:** In long-term mild diabetic pregnant group presented decrease in fast fiber number and area, increase in slow fiber area and also centrally myonuclei, sparse T tubes and several areas with disruption of sarcomeres. The short-term severe diabetic pregnant group presented increase slow fiber number, decrease fast fiber area, increase of subsarcolemmal and intermyofibrillar mitochondria and presence myelin figures. **Conclusion:** Diabetes in pregnant rats is a systemic skeletal muscle wasting disease with two myopathy types: long-term mild diabetic pregnant rats is selectivity skeletal muscle wasting and short-term severe diabetic pregnant rats the fiber type plasticity is suggested.

Key-words: diabetes, rats, extracellular matrix, skeletal muscle.

INTRODUCTION

Diabetes-related chronic hyperglycemia is associated with damage in different tissues, among them, the lower urinary tract and the striated skeletal muscle, the main organ involved in the glucose metabolism. Besides the contractility and movimentation capacity, the striated muscle is related to the capture and storage of glucose (1,2). It is composed by muscle fibers, connective tissue and extracellular matrix components (3). The striated skeletal muscle is a heterogeneous tissue composed by fiber types which differ according to their contractile and metabolic properties, the type I fibers (slow fiber) from oxidative system and slow contraction, the type II fibers, from fast contraction (fast fiber) from glycolytic system. The functioning and morphology of skeletal muscle depends on the proper interaction between contractile and not contractile components in this tissue (4,5). The mechanisms by which hyperglycemia contributes to changes in skeletal muscle has been widely studied, but what is structured in the scientific community is that the DM induces functional, metabolic and structural changes in skeletal muscle, caused by diabetic myopathy (6,7).

A high prevalence of UI and decreased vaginal squeeze pressure two years post-cesarean delivery among women with gestational diabetes mellitus compared to normoglycemic was detected (8). In translational researches, diabetic myopathy in urethral striated muscle of diabetic pregnant rats were detected. Fast and slow fiber profiles of pregnant rats with short-term severe diabetes (blood glucose> 300mg/dL) (9) and long-term mild diabetes (blood glucose between 120-300mg/dL) (10) induced by *streptozotocin* (STZ) were evaluated. The urethral striated muscle was thin, atrophic, disorganized, and disrupted. They were associated with fiber type switch and the predominance loss of the expression of fast fibers and higher expression of slow fibers. Long-term mild and short-term severe diabetes during pregnancy in rats affects the structural, ultrastructural, pathological and

immunohistochemical expression. These findings, together, suggest that the pelvic floor muscle (PFM) dysfunction, detected in clinical investigation may be attributed, in part, to the changes in urethral striated muscle of diabetic pregnant women (9,10).

Not only the urethral muscle but also the abdominal muscles take part on the urinary continence mechanisms (11,12). Several research groups have found a co-contraction mechanism in healthy volunteers during close-to maximum PFM contractions of different abdominal muscles (AM), among them, tranversus abdominis, external oblique, internal oblique and rectus abdominis (13).

Therefore, we hypothesized that DM associated with pregnancy in rats, leads to similar changes in other muscles and extracellular matrix involved in the urinary continence mechanisms. In the present study, to test this hypothesis we analyzed in an integrated way the effects of long-term mild (blood glucose between 120-300mg/dL) and short-term severe (blood glucose> 300mg/dL) STZ-diabetes in pregnant rats on the structure and ultrastructure of fiber muscles and extracellular matrix in the rectus abdominis muscle. Furthermore, the changes in the rectus abdominis muscle and urethral striated muscle in diabetic pregnant rats with two different glycemic levels and duration of hyperglycemia insult were also compared.

METHODS

Approval for this study was obtained from the Institutional Animal Care and Use Committee (process number 1003-2013) at the Botucatu Medical School/ Univ. Estadual Paulista - UNESP. Male and female Wistar rats (12-13 weeks, 250-300 g) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB, UNICAMP, Campinas, São Paulo, Brazil). They were housed in collective polypropylene cages (4 animals per cage) covered with metallic grids under controlled conditions of room

temperature (22±2ºC), humidity (55±5%), 12-hour light–dark cycle, and provided with food and water *ad libitum*.

Experimental Design

Seventy eight rats were randomly allocated into six groups: the virgin group (n=13), the pregnant group (n=13), the long-term mild diabetic group (n=13), the short-term severe diabetic group (n=13), the long-term mild diabetic pregnant group (n=13) and the short-term severe diabetic pregnant group (n=13). The experimental sequence of all groups is showed in Figure 1. All animals were killed on 133th day of life.

Long-term mild diabetes. Parental non-diabetic female rats were mated with non-diabetic males to obtain female newborns. For the induction of long-term mild diabetes (blood glucose between 120-300 mg/dL), female newborns received *streptozotocin* (SIGMA Chemical Company, St. Louis, MO, USA), a beta (β)-cytotoxic agent, diluted in citrate buffer (0.1 M; pH 4.5) at a dose of 100 mg/kg on the first day of life by subcutaneous route (14). Newborn rats remained with their mothers until day 21 of life (weaning period). Glucose tolerance test (GTT) was performed in the 129th day of life in the virgin and long-term mild diabetic groups and in the 17th day of pregnancy in pregnant and in long-term mild diabetic pregnant group, to assess the development of altered glucose metabolism and the results was used as a inclusion criteria in certain groups. Blood glucose concentrations were measured by a One-Touch Ultra glucometer (LifeScan, Johnson and Johnson®, Milpitas, CA, USA) and the values were expressed in mg/dL. Rats with glycemic levels higher than 140 mg/dL in more than two measures during GTT was included as moderate diabetes (Figure 1 – Annex). Other female newborns received only citrate buffer and these animals were considered as non-diabetic and included in this study as a virgin group.

Short-term severe diabetes. The diabetes was induced in the adult life of female rats (90 days of age) by STZ injection (SIGMA Chemical Company, St. Louis, MO, USA). The STZ was administered intraperitoneally at 50 mg/kg to produce a permanent and severe diabetic state. Blood samples were taken 72 h after STZ injection to confirm severe diabetes (blood glucose level >300 mg/dL) (15). Other female rats received only citrate buffer and these animals were considered as non-diabetic and included in this study as a virgin group.

Mating period. The female rats randomly selected for pregnancy in adult life (all pregnant groups) were maintained overnight with non-diabetic male rats. The morning of the next day, the presence of spermatozoa in the vaginal smear was designated as gestational day 0.

Tissue Harvesting

The pregnant rats were euthanatized on day 21 of pregnancy and the non-pregnant rats on a similar/equivalent day, by sodium thiopental (Thiopentax®, 80mg/kg dose). At the time of Cesarean section, it were obtained fragments of the lower-third rectus abdominis muscle.

Histological examination, immunohistochemical stain, and morphometric analysis (n=10 samples/group)

The rectus abdominis muscle was exposed, dissected, removed, reduced the edges, wrapped in talc and frozen in liquid nitrogen and kept at -80°C. The frozen muscle specimens were cut into 10-µm-thick cross-sections using a cryostat (Leica CM 1800). The cross-sections were fixed on microscope glass slides in cold acetone for 10 minutes and were stained for hematoxylin and eosin (H&E), picrosirius red and immunohistochemical. The slides were examined by light microscopy and photographed.

Hematoxylin and eosin. Slides stained with H&E were considered the reference standard of a normal rectus abdominis structure and used to observe the general morphology of the striated muscle.

Picrosirius red. This color-segmentation method was employed to determine the tissue area of stained red (collagen) and yellow (muscle fiber) in the same section (Figure 2). For this analysis 10 sections/animal for quantitative morphometric analysis of collagen area and muscle area (600 Picrosirius red-stained images, 20× magnification) were used and the Image Pro Plus 7.0 image analysis software (Media Cybernetics, Ins. USA) at Case Western Reserve University (USA) were performed. The software can distinguish regions stained with different colors and accurately measure the different areas by counting the pixels and converting pixels to area (μm^2) (Figure 2).

Immunohistochemistry. For staining of fast and slow-type skeletal muscle fibers, the sections were incubated with antibodies directed towards WB-myosin heavy chain, fast (WB-MHCf) Novocastra (1:120) and WB-MHC slow (WB-MHCs) Novocastra (1:180). A quantitative method was performed for type fiber area. The area was determined by measuring ~ 200 muscle fibers from each animal, also, fiber type number in 4 sections/animal were counted with Image Processing and Analysis in Java software (ImageJ version 1.36b, National Institutes of Health, Bethesda, MD, USA). The software can measure and count each fiber area defined manually and convert the pixels of the image in area (μm^2) (Figure 3).

Ultrastructure ***(n=3 sample/group)***

The samples were immersed in the karnovsky solution during 24 hours for fixation before post fixation in osmium tetraoxide. After, the specimens were post fixed in osmium

tetroxide and embedded in epoxy resin. The sections were obtained by ultratome and the stained sections were examined by transmission electron microscopy (TEM) (Philips CM 100).

Statistical analysis

To avoid overinfluence of data from a single group, analysis of muscle area, collagen area, muscle/collagen ratio, fast/slow area, ratio and number, the two-way analysis of variance was made (16). Results are expressed as means ± SD. For the collagen and muscle area Student's t-test were performed and for the ratio muscle/collagen, the gamma model was used. For the fiber type number the Poisson test the ratio fast/slow gamma model was performed. For fiber area a conversion on square root to normalize the data was previously made and after Student's t-test was performed. $P < 0.05$ was considered significant. All analyzes were performed using the SAS software for Windows v.9.2.

RESULTS

General characteristics

General physical characteristics of the rats (body weight, body weight gain during pregnancy and blood glucose levels) in this study were compatible with long-term mild and short-term severe diabetes (Table 1). The short-term severe diabetes groups presented glycemic values >300mg/dL and the long-term mild diabetes groups presented similar levels to virgin and pregnant groups but two or more points up to 140mg/dL in the GTT (Figure 1- Annex). The weight gain during pregnancy were lower in long-term mild and short-term severe diabetes compared to pregnant group.

Rectus abdominis muscle characteristics

The H&E transverse sections of the rectus abdominis muscle showed fibers from

different sizes, polygonal format and peripheral myonuclei. Each one was surrounded by connective tissue and involving a group of fibers, a thick layer of connective tissue (Figure 4).

Muscle and collagen morphometric analysis area

The photomicrographs of rectus abdominis of female rats of the six groups were showed in Figure 5. Data from muscle area, collagen area and muscle/collagen ratio of 6 groups were presented in Table 2. A significant decrease of muscle area in short-term severe diabetic pregnant group compared to virgin, short-term severe diabetic and pregnant groups were detected. There were a significant increase in collagen area in pregnant group compared to virgin group. The muscle/collagen ratio was significant lower in the pregnant group compared with virgin group and in the short-term severe diabetic pregnant group compared with virgin group (Figure 5). The long-term mild pregnant group presents similar results to pregnant and to short-term severe diabetic pregnant group.

Immunohistochemical fiber type number

The fiber type number and ratio data was showed in the Table 3. The long-term mild diabetic group compared by two way analysis, with virgin, long-term mild diabetic and pregnant presented a significant lower number of fast fiber and higher number of slow fibers, and so the fast/slow ratio was significantly smaller. In short-term severe diabetic pregnant group the number of slow fiber was the highest compared to all other groups. The number of fast fiber was higher in short-term severe diabetic pregnant group compared to long-term mild diabetic pregnant group and short-term severe diabetic pregnant group. The fast/slow ratio in short-term severe diabetic pregnant group is lower compared to virgin, short-term severe diabetic and pregnant groups (Table 3 Figure 6).

Immunohistochemical morphometric analysis area

The long-term mild diabetic pregnant group presented small fast type area compared

to virgin, pregnant and long-term mild diabetic groups and higher slow fiber type area compared to virgin and long-term mild diabetic groups. The short-term severe diabetic pregnant group by two-way analysis presented the smallest fast fiber area and higher slow fiber area compared to virgin and short-term severe diabetic groups. The pregnant group showed smaller fast fiber area and higher slow fiber area compared to virgin group (Figure 7).

Ultrastructural analysis

In the ultrastructural analysis, the rectus abdominis muscle of the virgin group showed well-organized myofibrils forming intact sarcomeres and organized triad, a system formed by sarcoplasmic reticulum and t tuble, with morphological characteristics related to different muscle fiber types and normal distribution in the intermyofibrillar mitochondria. The pregnant group presented a disorganized Z line and thinning sarcomere. Instead the long-term mild diabetic group presented well-organized myofibrils and myelin figures that was related to degenerated organelles. The short-term severe diabetic showed numerous subsarcolemmal and intermyofibrillar mitochondria and also a thinning in striated muscle cells. In long-term mild diabetic pregnant group central myonuclei, sarcoplasmic reticulum sparse, dilated T tubes and areas with disruption of sarcomeres were present (Figure 8).

Comparison between the changes in the rectus abdominis muscle and urethral striated muscle in diabetic pregnant rats with two different glycemic levels

Table 4 shows the summary of morphological changes in rectus abdominis muscle compared to changes in urethral striated muscle in severe and mild diabetes pregnant rats obtained by two different papers from our research group (9,10,17). The results showed that in both urethral striated muscle and rectus abdominis, long-term mild diabetic pregnant rats and short-term severe diabetic pregnant rats showed decreases in fast and increased in slow fiber type. The increase in collagen area detected in urethral striated muscle of long-term

mild and short-term severe diabetic pregnant rats was not detected in rectus abdominis of both groups.

DISCUSSION

Years of investigation have led to an increase understanding of the mechanisms of additive or synergistic effects on post-partum UI either in diabetic women or in diabetic rats. A number of hypotheses have been put forth trying to explain in translational studies a pragmatic way to increase the level of scientific evidence with more reliable understanding of a positive relationship between GDM and UI.

This study demonstrated that diabetic pregnant rats had important alterations on the fiber skeletal striated muscles structure and ultrastructure. Differences in fiber type composition and fiber area were detected in these integrated morphological analysis of the effects of long-term mild and short-term severe diabetes in pregnant rats. The rectus abdominis muscle of long-term mild diabetic pregnant group presented the lowest fast fiber number, the highest slow fiber area and also centrally myonuclei, sarcoplasmic reticulum well developed, sparse T tubes and several areas with disruption of sarcomeres. All these alterations putting together characterize a tissue with selective muscle wasting. In contrast, the short-term severe diabetes pregnant rats presented the highest slow fiber number, the smallest fast fiber area, an increase of subsarcolemmal and intermyofibrillar mitochondria, presence myelin figures and local areas with sarcomere disruption and a significant decrease in the muscle/collagen ratio compared to virgin group. These results suggest that in short-term severe diabetic pregnant group there is a muscle adaptation with fiber type plasticity.

Muscle mass is dependent upon the relative balance of fiber biosynthesis versus fiber degradation (18). Apoptosis is an essential part of skeletal muscle development and homeostasis (19) however, dysregulation is associated with muscle atrophy (20). The

dysregulation can be caused from several factors, such as innervation disorders, physical activity reduction, ageing, and metabolic abnormalities (especially in proteins, carbohydrates, and lipids) (21,22). The different conditions leading to muscle loss involve different cell signaling pathways that might lead to programmed cell death (apoptosis), increased protein breakdown, or even decreased activation of the satellite cells responsible for muscle regeneration (23). Diabetes is associated with accumulation of reactive oxygen species which contributes to diabetic myopathy by upergulation of atrophy-related genes (termed 'atrogenes'), concomitant with a decrease in genes associated with muscle growth (24,25). Our results demonstrated, in long-term mild diabetic group, an important decrease in fast fibers area. A fiber type selectively skeletal muscle wasting appears to be attributed to different signaling pathways, and most of them are relevant to abnormality of protein degradation of fast fiber type, although the specificity of these signals are still under debate. (18). Despite muscle wasting, contractility properties were not altered when expressed relative to muscle mass, suggesting that metabolic changes within muscle fibers precede detectable impairments in contractile properties (26)

Both of our study groups exhibited an increment in the slow fibers. Similar changes in the normal architecture of muscles who underwent a hyperglycemia environment have been largely found in many researches (26-29). In our study, a sparse transverse tubules and sarcoplasmatic reticulum in long-term mild diabetic pregnant group were found. These changes could be related to the reduced skeletal muscle oxidative phosphorylation and to disorders in calcium homeostasis as described recently (30). An enhanced calcium pump activity of sarcoplasmic reticulum in diabetic rat skeletal muscle has been well established (31). These fibers exposed to hyperglycemic environment exhibited swollen transverse tubules and the altered calcium handling that may influence the fiber type switching (32,33).

Skeletal muscle is a complex, versatile tissue composed of a large variety of

functionally diverse fiber types with an extraordinary adaptive potential (34). Individually, the muscle fibers have differences in contraction velocity, oxidation, capillarity and number and size of mitochondria (35). The intertissue cross-talk plays an important role in the maintenance of normal glucose homeostasis (26,36). Skeletal muscle displays enormous plasticity and the fiber composition can be regulated according to physiological properties and biochemical action such as innervation/neuromuscular activity, electrical activity exercise training, mechanical loading/unloading, hormones, aging, DM and play important roles in the regulation of muscle fiber composition (29,34). Our short-term severe diabetic pregnant study group, seems to change the profile in order to maintain the glucose homeostasis with a decrease in fast fiber area, increase in the slow fiber number and therefore an increase in the mitochondria number. Muscle is the main organ of glucose uptake and the glucose transporter proteins (GLUT4) which is more expressed in the slow fibers and related to the oxidative capacity mediate the transport across the cell membrane of skeletal muscle (37). Thus, it is likely that slow fibers are more important than fast fibers for maintaining glucose homeostasis (38-40).

Taken together our findings and the results from urethral striated muscle (9,10,17), either urethral striated muscles or rectus striate abdominal muscles presented similar characteristics in severe short-term and in moderate long-term diabetic pregnant rats. Long-term mild diabetes showed skeletal muscle wasting, increase in slow fibers and decrease in fast fibers characterizing the diabetic myopathy and severe short-term diabetic pregnant rats showed selective fiber type plasticity. These different aspects of diabetic myopathy appears to be more intense with long-term low glucose levels than with exposure to short-term high levels of hyperglycemia. The similar changes in striated rectus abdominis muscle as well as in striated urethral muscle in diabetic pregnant rats is the major novel finding of the current study.

Regarding to collagen area there is a difference between urethral striated muscle and rectus abdominis. In this study there was no difference in collagen area of rectus abdominis muscle between groups, however an increase in the collagen area in urethral striated muscle of long-term mild diabetic pregnant group and an alteration in collagen composition (collagen type I/III ratio) in short-term diabetic pregnant group were found in urethral striated muscle (9,10,17). The basis of these discrepancies is not clearly understood, although it may involve the differences in the composition of both tissues. The striated urethral muscle has close contact with urothelium and smooth muscle and rectus abdominis is a unique muscle tissue. Diabetes seems to be related with increased collagen (41-42) meanwhile studies have investigated alterations in the connective tissue in urinary incontinence following different lines of research. Some authors correlated UI with decreased collagen fibers, while others correlated with and increased of collagen without no well-accepted conclusion of whether UI correlates with collagen (43-44). Instead of the differences in collagen our results confirmed DM associated with pregnancy in rats as a systemic disease since leads to similar changes in muscles involved in the urinary continence mechanisms.

Diabetic myopathy is a term used to characterize a failure to maintain healthy muscle. This problem is believed to contribute to the progression of complication due to the vital importance of skeletal muscle for physical and metabolic wellbeing (45). The goal of the present study was to gain a more comprehensive understanding of this diabetic myopathy providing the importance of two different glycemic levels and two different durations of hyperglycemic stimulus on skeletal muscle. In each diabetic pregnant models, a different failure to maintain healthy muscle was observed, and could be termed diabetic myopathy. Diabetic skeletal muscle disease or “myopathy”, a much less studied complication of poorly controlled diabetes, is also a common clinical condition

characterized by lower muscle mass, weakness and an overall reduced physical capacity (46-48). In diabetic myopathy, the most commonly finding is a loss of peak contractility force, likely a result of the atrophy or impaired growth of the glycolytic muscle fibers and exists in the absence of observable neuropathic complications (49,50).

This impact of diabetes mellitus in pregnancy on skeletal muscle healthy confirms the negative influence of hyperglycemic levels and long-term duration of mild glycemic levels. Identifying the differences in the proponents that could attenuate the normal muscle function in gestational diabetes will lead to development of therapies that could restore the muscle healthy and attenuate the UI in diabetic pregnant woman.

The present study has comprehensive characterized diabetes in pregnancy as a systemic skeletal muscle disease with two different myopathy types: in a long-term mild diabetic pregnant rats a selective skeletal muscle wasting is a current finding and in short-term severe diabetic pregnant rats the fiber type plasticity is demonstrated.

The results of our study should be interpreted by awareness of the following limitations. Given the known effects of STZ on skeletal muscle, interpretation of our results using STZ-induced diabetic model should be made cautiously. Rats are quadrupeds and they have tails with associated musculature. Future animal studies should consider other alternative models to study the contractility, metabolic and phenotypic propriety in STZ diabetic muscle concerns of the current state of knowledge regarding the effects of diabetes on skeletal muscle (26,51).

In conclusion, diabetes in pregnant rats is a systemic skeletal muscle wasting disease with two different myopathy types: one as a time dependent disorder and other as a hyperglycemic levels disorder. In long-term mild diabetic pregnant rats the selectivity skeletal muscle wasting is a current finding and in the short-term severe diabetic pregnant rats the fiber type plasticity is suggested. As skeletal muscle is a primary organ of glucose

disposal and an individual physical capacities are tightly coupled to their muscle mass, understanding the basic mechanisms underline diabetic myopathy as a systemic disease has tremendous importance to the development of appropriate and successful long-term therapeutic strategies to improve quality of life and reduce overall health care costs (26).

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COMPETING INTERESTS

The authors declare that there are no competing interests.

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recovery from urinary incontinence due to simulated childbirth in female rats. American journal of physiology Regulatory, integrative and comparative physiology. 2007; 293(2): R950-5.

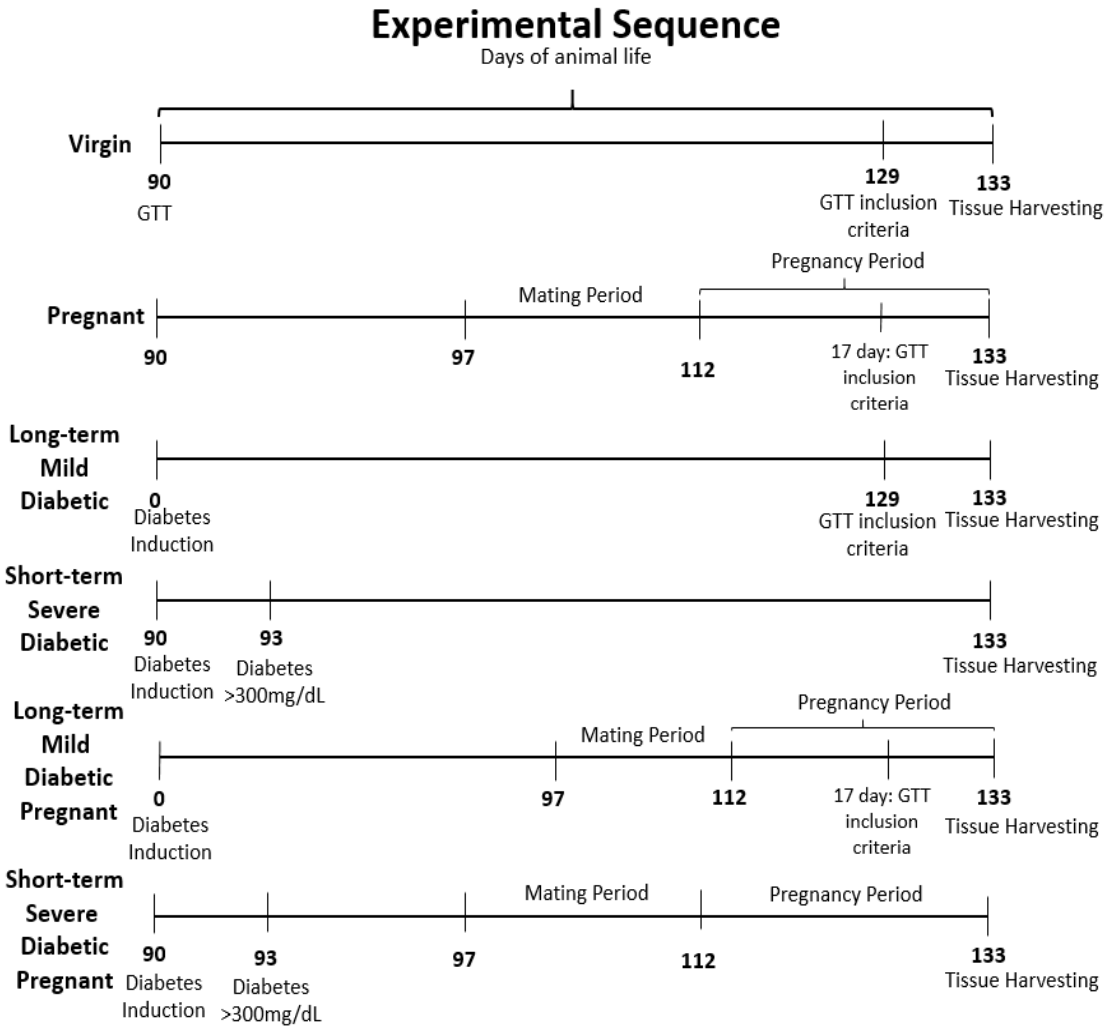

Figure 1. Experimental sequence of all groups.

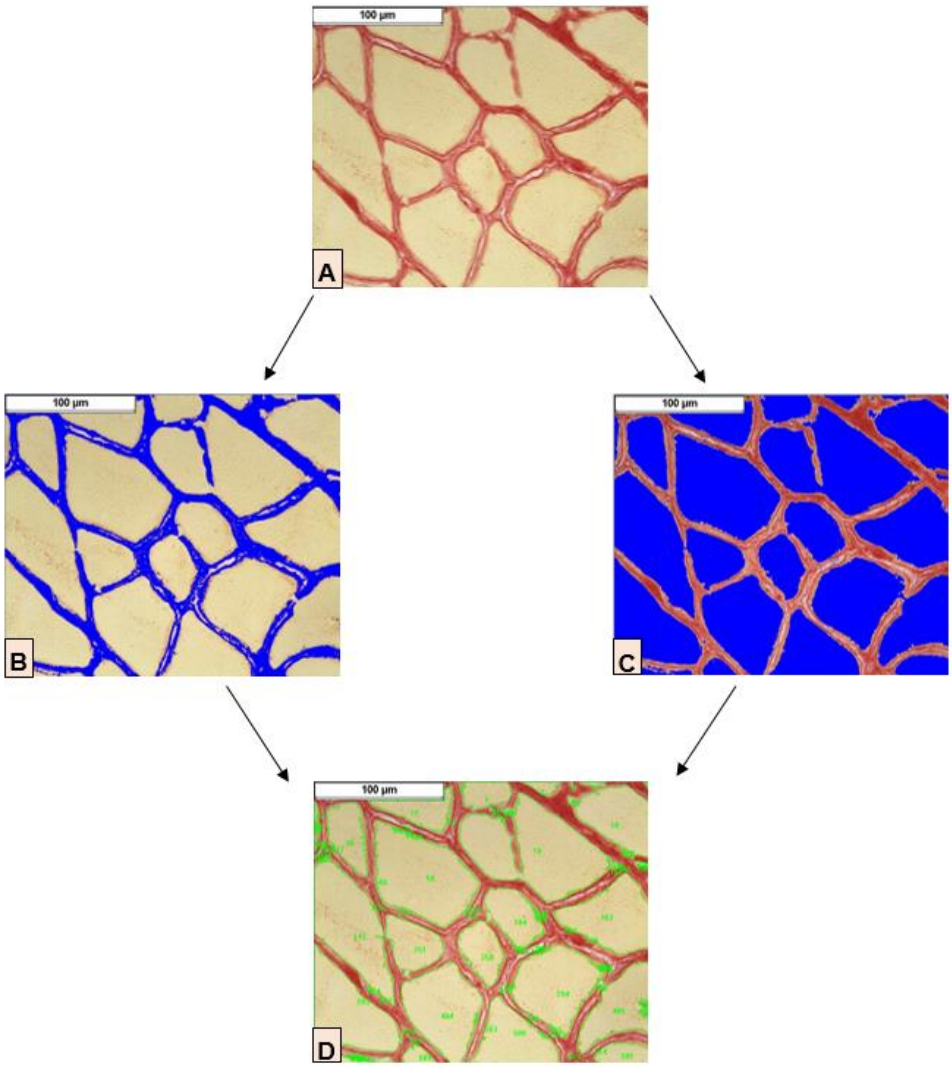

Figure 2. Image analysis of a picrosirius red-stained transverse section of a rectus abdominis of rat, quantitativly analyzed by Image Pro Plus 7.0 image software. Striated muscle (yellow) and collagen (red) (A) were recognized and captured by automated digital image analyzer for measurement of total tissue cross-sectional area. The section demonstrating measurement of (B) total area of collagen and muscle (C) highlighted by the software and (D) the pixels areas converted to μm^2. Scale bars= 100 µm.

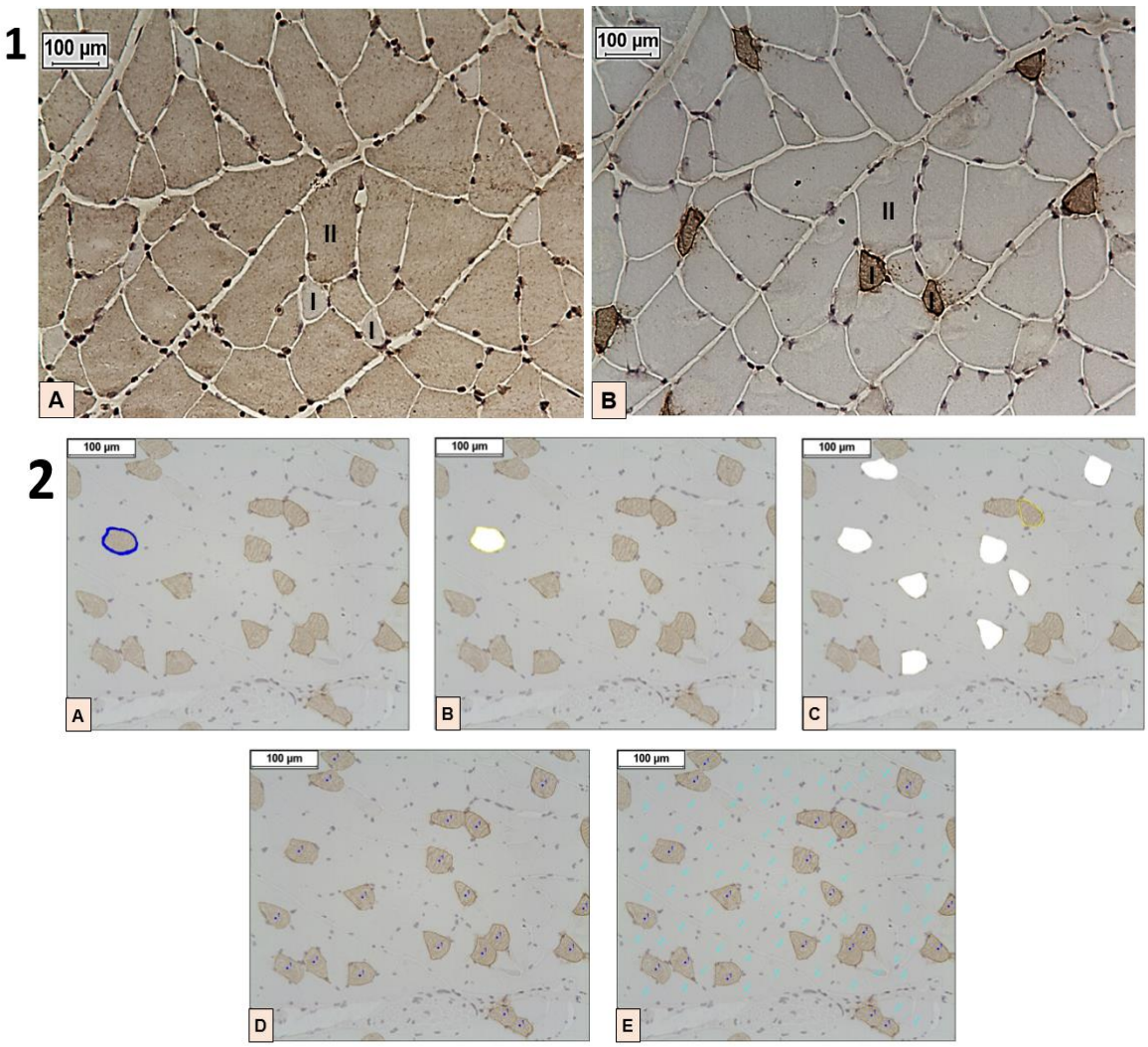

Figure 3. 1 - Image analysis after immunohistochemistry for fast (A) and slow (B) fibers, transverse section of a rectus abdominis of female rat. 2 - quantitativly analysis by Image Processing and Analysis in Java software. The section demonstrating measurement of a single fiber (A and B) several fibers (C). The delimited area was selected mannualy then the software translate the area in pixels to μm^2. Also with the software, the total number of fast/slow fibers (D and E) was counted. Scale bars= 100 µm.

Table 1. The effect of long-term mild diabetes ans short-term severe diabetes on changes in body weight, maternal weight gain and glucose.

	Virgin	Long-term Mild Diabetic	Short-term Severe Diabetic	Pregnant	Long-term Mild Diabetic Pregnant	Short-term Severe Diabetic Pregnant
Body weight	244,26 ±36,32	243,32 ±25,49	225,55 ±29,46	374,61 ±30,58	349,39 ±38,03	299,94 ±26,74[1 2]
Maternal Weight Gain	-	-	-	117,28 ±27,44	98,76 ±29,95	71,31 ±33,13[1 2]
Glucose	100,87 ±6,24	107,37 ±18,03	517,30 ± 84,91[1 2]	87,17 ±8,50	86,06 ±27,67	511,25 ±71,04[1 2]

Values are means ± SD

[1] $p<0,05$- Significantly diferent from virgin group.

[2] $p<0,05$- Significantly diferent from long-term mild diabetic.

[3] $p<0,05$- Significantly diferent from pregnant group.

[4] $p<0,05$- Significantly diferent from long-term mild diabetic pregnant group.

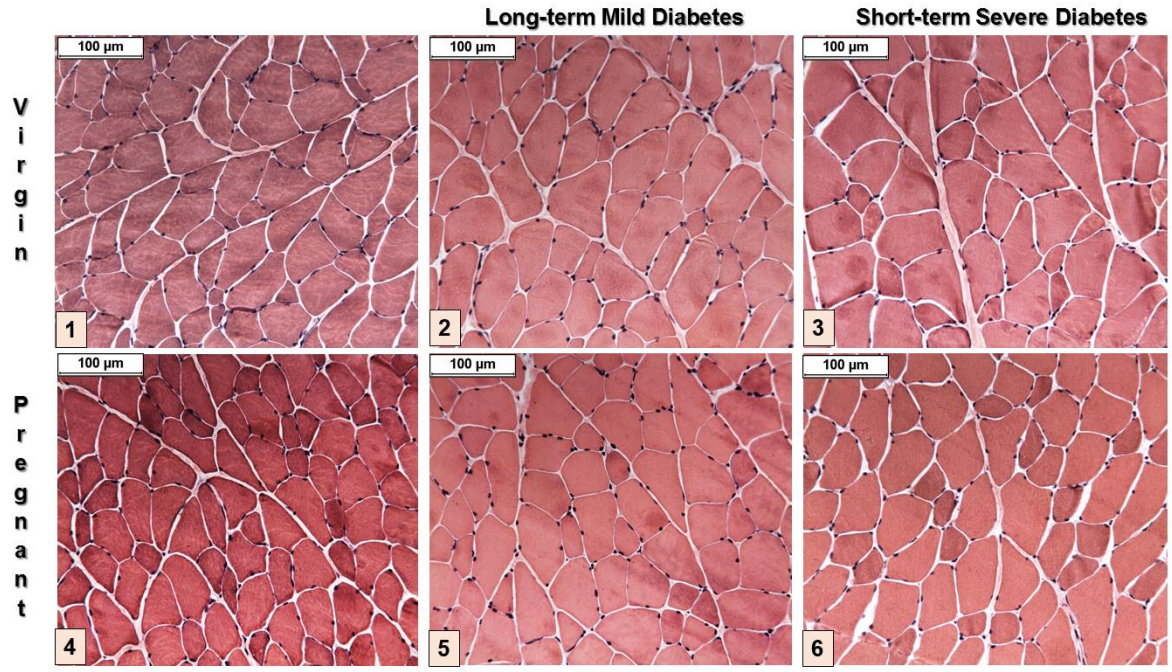

Figure 4. Histological transverse sections of the rectus abdominis muscle of female rats photomicrographs from one representative animal of each group. Virgin (1), Long-term Mild Diabetic (2), Short-term Severe Diabetic (3), Pregnant (4), Long-term Mild Diabetic Pregnant (5) and Short-term Severe Diabetic Pregnant (6). H&E stained. Magnification x 20. Scale bars = 100 µm.

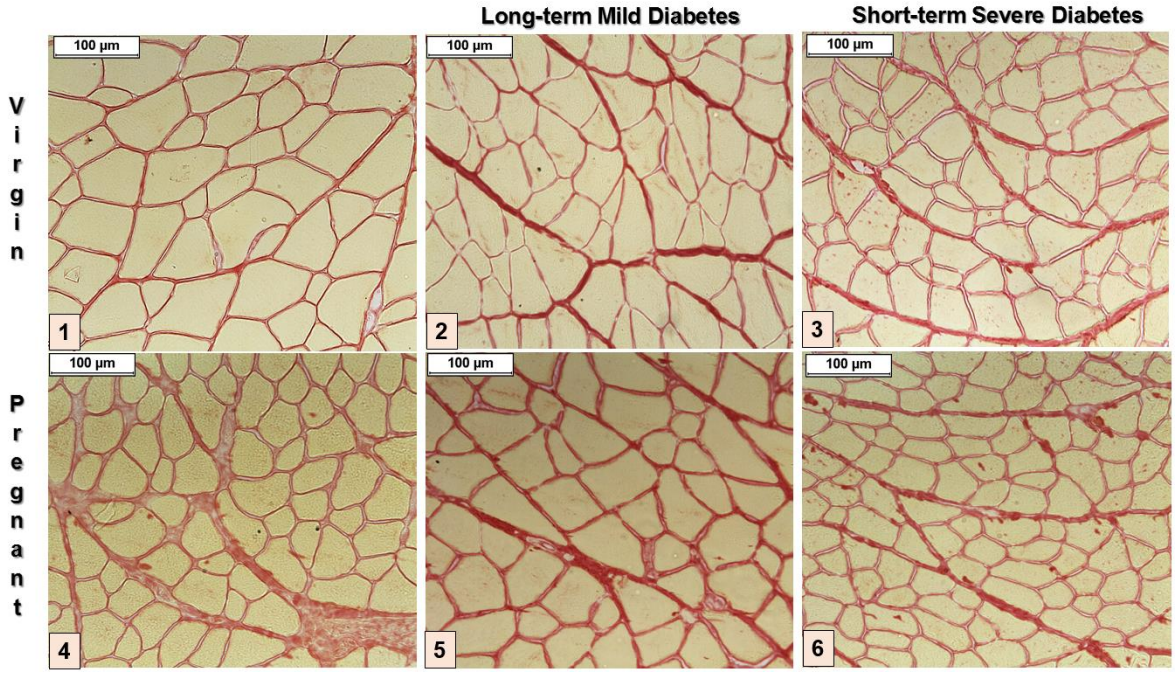

Figure 5. Photomicrographs of rectus abdominis of female rat after picrosirius red-stained. Virgin (1), Long-term Mild Diabetic (2), Short-term Severe Diabetic (3), Pregnant (4), Long-term Mild Diabetic Pregnant (5) and Short-term Severe Diabetic Pregnant (6). Magnification x20. Scale bars = 100 µm.

Table 2. The effect of long-term mild diabetes and short-term severe diabetes on changes in collagen, muscle area and muscle/collagen ratio between the groups.

	Virgin	Long-term Mild Diabetic	Short-term Severe Diabetic	Pregnant	Long-term Mild Diabetic Pregnant	Short-term Severe Diabetic Pregnant
Muscle Area	169962±13028,59	164232,76±15229,23	157744,62±21233,23	170115,6±10857,80	166347±17470,50	163825,48±17248.38**bcd**
Collagen Area	29460,8±10146,37	32405,42±12270,97	33963,92±11734,08	32534,78±9082,36**a**	32342,8±11795,77	31575,62±8580,35
Muscle/Collagen	6,75±3,14	6,14±3,24	5,38±2,35	5,89±2,64**a**	6,25±3,37	5,64±1,89**b**

The results are expressed in mm^2. Values are means ± SD (t test)
a $p < 0,05$ – Significantly difference between groups virgin and pregnant.
b $p < 0,05$ – Significantly difference between groups virgin and short-term severe diabetic pregnant.
c $p < 0,05$ – Significantly difference between groups short-term severe diabetic and short-term severe diabetic pregnant .
d $p < 0,05$ – Significantly difference between groups pregnant and short-term severe diabetic pregnant.

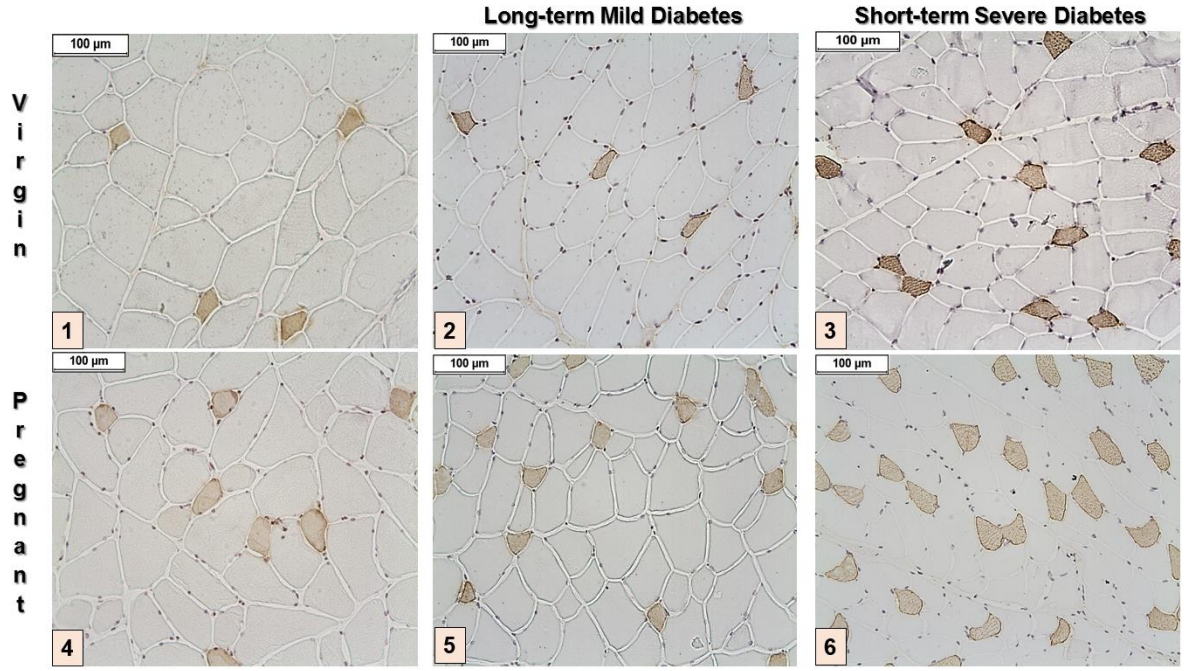

Figure 6. Images of immunohistochemistry for slow fibers, transverse section of a rectus abdominis of female rats. Virgin (1), Long-term Mild Diabetic (2), Short-term Severe Diabetic (3), Pregnant (4), Long-term Mild Diabetic Pregnant (5) and Short-term Severe Diabetic Pregnant (6). Magnification x20. Scale bars = 100 µm.

Table 3. The effect of long-term mild diabetes and short-term severe diabetes on changes in fast and slow fiber type number and fast/slow ratio of rectus abdominis muscle.

	Virgin	Long-term Mild Diabetic	Short-term Severe Diabetic	Pregnant	Long-term Mild Diabetic Pregnant	Short-term Severe Diabetic Pregnant
FAST	64,8±10,56	63,98±12,82	56,38±8,85	64,05±13,35	56,75±6,25**abc**	62,05±13,68**eg**
SLOW	9,13±3,01	10,4±2,81	13,28±3,44	8,98±4,38	16,4±5,08**abc**	19,75±7,52**defg**
FAST/SLOW	7,77±2,51	6,64±2,33	4,51±1,25	8,91±4,47	3,86±1,46**abc**	3,57±1,42**def**

Values are means ± SD (t test)
a $p < 0,05$ – Significantly difference between groups virgin and long-term mild diabetic pregnant.
b $p < 0,05$ – Significantly difference between groups long-term mild diabetic and long-term mild diabetic pregnant.
c $p < 0,05$ – Significantly difference between groups pregnant and long-term mild diabetic pregnant.
d $p < 0,05$ – Significantly difference between groups virgin and short-term severe diabetic pregnant.
e $p < 0,05$ – Significantly difference between groups short-term severe diabetic and short-term severe diabetic pregnant.
f $p < 0,05$ – Significantly difference between groups pregnant and short-term severe diabetic pregnant.
g $p < 0,05$ – Significantly difference between groups long-term mild diabetic pregnant and short-term severe diabetic pregnant.

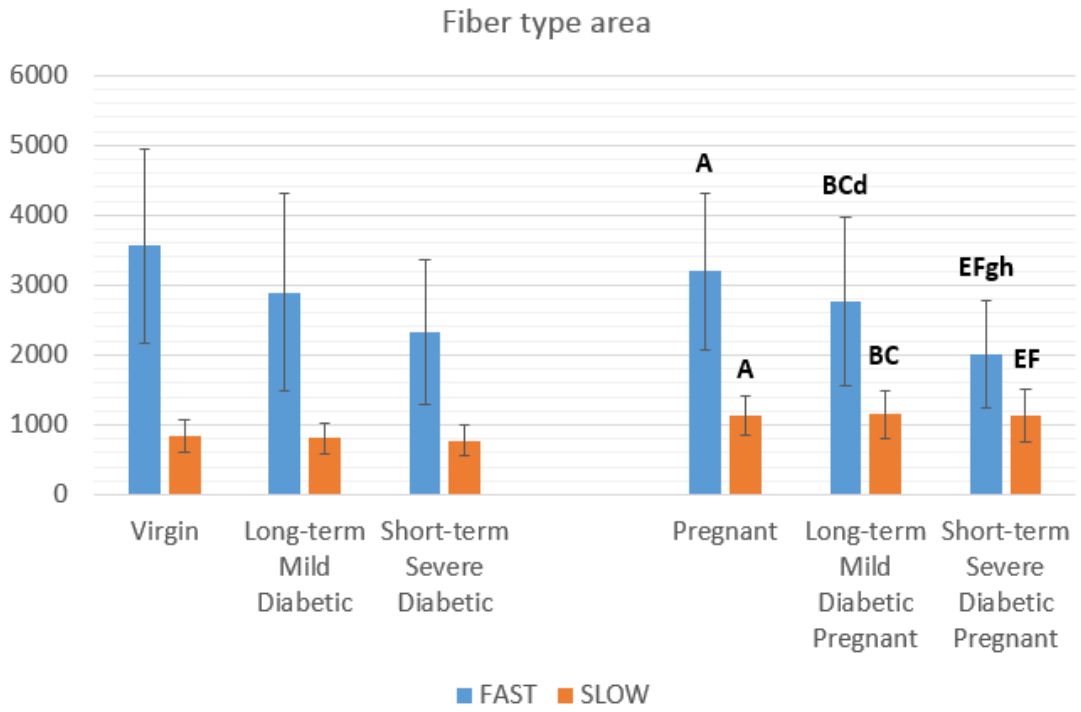

Figure 7. Changes in fiber type area of each group at the end of experimental period.
A $p < 0,05$ – Significantly difference between groups virgin and pregnant.
B $p < 0,05$ – Significantly difference between groups virgin and long-term mild diabetic pregnant.
C $p < 0,05$ – Significantly difference between groups long-term mild diabetic and long-term mild diabetic pregnant.
d $p < 0,05$ – Significantly difference between groups pregnant and long-term mild diabetic pregnant.
E $p < 0,05$ – Significantly difference between groups virgin and short-term severe diabetic pregnant.
F $p < 0,05$ – Significantly difference between groups short-term severe diabetic and short-term severe diabetic pregnant.
g $p < 0,05$ – Significantly difference between groups pregnant and short-term severe diabetic pregnant.
h $p < 0,05$ – Significantly difference between groups long-term mild diabetic pregnant and short-term severe diabetic pregnant.

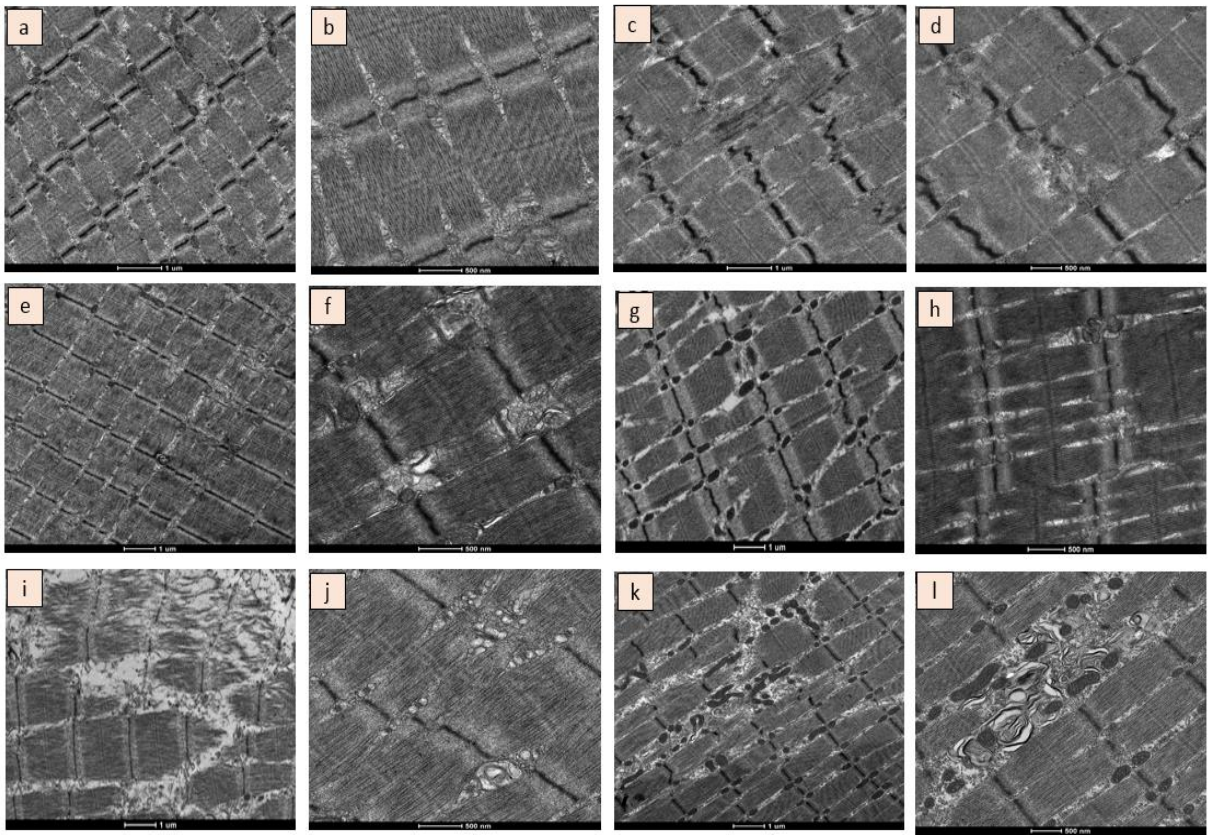

Figure 8. Electron micrographs of rectus abdominais muscle fibers from a, b virgin, c, d pregnant, e, f long-term mild diabetic, g, h short-term severe diabetic, i, j long-term mil diabetic pregnant and k,l short-term severe diabetic pregnant.

Table 4. Comparison between the changes in the rectus abdominis muscle and urethral striated muscle in diabetic pregnant rats with two different hyperglycemic models.

	Rectus abdominis		Urethral striated muscle	
	Long-term Mild Diabetic Pregnant	**Short-term Severe Diabetic Pregnant**	**Long-term Mild Diabetic Pregnant (Piculo et al., 2014)**	**Short-term Severe Diabetic Pregnant (Marini, 2014)**
Collagen area	No difference	No difference	↑ ↑	↑ I/III
Striated muscle area	↓	↓ ↓	↓	↓ ↓
Fiber type number	↓ ↓ FAST ↑ SLOW	↓FAST ↑ ↑ SLOW	↓ FAST ↑ SLOW	↓ FAST ↑ SLOW
Fiber type area	↓FAST ↑ ↑ SLOW	↓ ↓ FAST ↑ ↑ SLOW	_	_

ANNEX

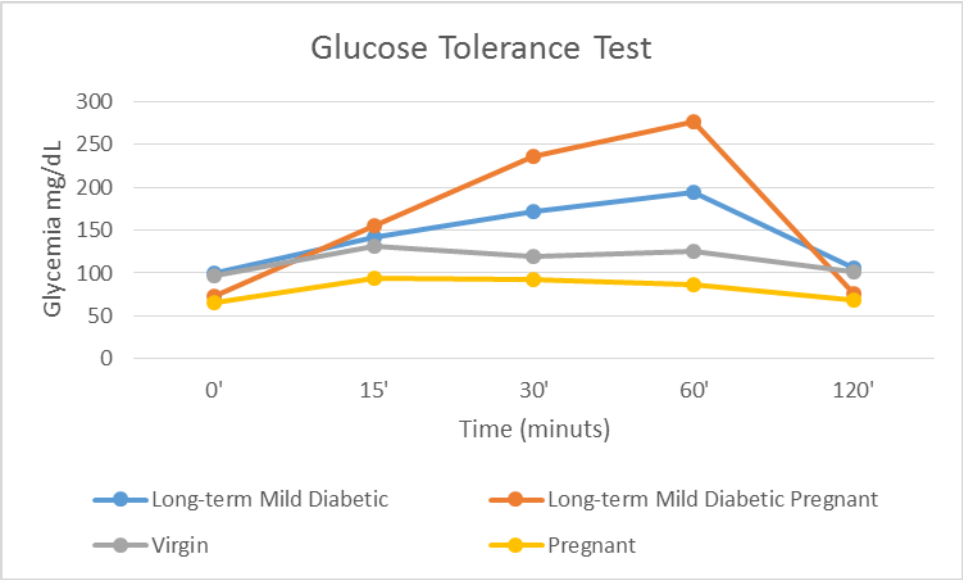

Figure 1. Glucose tolerance test of the groups: virgin, pregnant, long-term mild diabetic and long-term mild diabetic pregnant.

Anexos

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UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

FMB

CEUA Ética
Comissão de Ética no Uso de Animais

Criada através da Portaria DFM nº 611 de 13/12/2012

Certificado

CERTIFICAMOS que o (Protocolo CEUA 1003/2013) Análise morfológica do músculo reto abdominal de ratas prenhes diabéticas, a ser conduzido por Giovana Vesentini, orientada pela Profª. Titular Marilza Vieira Cunha Rudge, co-orientada por Angélica Mércia Pascon Barbosa, com a colaboração de Débora Cristina Damasceno, Fernanda Piculo, Gabriela Marini, Selma Maria Michelin Matheus e Sérgio Luis Felisbino, com o Apoio Técnico de Talísia Collachiti Moreto, está de acordo com o Conselho Nacional de Controle de Experimentação Animal - CONCEA, com a ressalva de que os "ratos" são provenientes de Biotério convencional, sem condições de atestar a Sanidade dos mesmos.

Projeto de Pesquisa aprovado em reunião da CEUA em 28/02/2013

Profª Drª Maria Rosa Bet Moraes Silva
Presidente da CEUA

Alberto Santos Capelluppi
Secretário da CEUA

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ANEXO 2 – Comprovante de envio para publicação

International Journal of Urology

INTERNATIONAL JOURNAL OF UROLOGY

SKELETAL MUSCLE WASTING AND FAST/SLOW FIBER TYPE PROFILE CHANGES CAUSED BY DIABETES IN PREGNANT RATS

Journal:	*International Journal of Urology*
Manuscript ID:	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Vesentini, Giovana; UNESP Botucatu Medical School, Gynecology and Obstetrics Marini, Gabriela; UNESP Botucatu Medical School, Gynecology and Obstetrics Piculo, Fernanda; UNESP Botucatu Medical School, Gynecology and Obstetrics Barbosa, Angelica; UNESP Botucatu Medical School, Gynecology and Obstetrics Damasceno, Débora; UNESP Botucatu Medical School, Gynecology and Obstetrics Calderon, Iracema; Unesp Botucatu Medical School, Gynecology and Obstetrics Matheus, Selma; UNESP Botucatu Biosciences Institute, Anatomy Felisbino, Sergio; UNESP Botucatu Biosciences Institute, Morphology Hijaz, Adonis ; Case Western Reserve University, Urology Rudge, Marilza; Unesp Botucatu Medical School, Gynecology and Obstetrics
Key Words:	diabetes, rats, extracellular matrix, skeletal muscle
Abstract:	Objectives: To analyzed in an integrated way the effects of DM in different intensities associated with pregnancy in the rectus abdominis muscle of rats and compare with previous results of urethral striated muscle. Methods: Two hyperglycemic levels was studied the long-term mild diabetes, when female newborns received streptozotocin (100 mg/kg body weight, sc route) and short-term severe diabetes (blood glucose level >300 mg/d) the adult animals received streptozotocin at 50 mg/kg, ip route. At day 21 of pregnancy the rats were euthanatized and the lower-third rectus abdominis muscle were harvested and analyzed by picrosirius red staining for collagen and muscle components, immunohistochemistry for fast and slow muscle fibers and transmission electron microscopy for ultrastructural analysis. Results: In long-term mild diabetic pregnant group presented decrease in fast fiber number and area, increase in slow fiber area and also centrally myonuclei, sparse T tubes and several areas with disruption of sarcomeres. The short-term severe diabetic pregnant group presented increase slow fiber number, decrease fast fiber area, increase of subsarcolemmal and intermyofibrillar mitochondria and presence myelin figures. Conclusion: Diabetes in pregnant rats is a systemic skeletal muscle