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Efeito do diabete induzido por *streptozotocin* na matriz extracelular e no músculo estriado uretral em ratas prenhes

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Epígrafe

“Somente depois de ter andado por terras estranhas
É que pude reconhecer a beleza de minha morada.
A ausência mensura o tamanho do local perdido
Evidencia o que antes estava oculto, por força do costume.
Olhei minha mãe como se fosse a primeira vez.
Olhei como se eu voltasse a ser criança pequena
A descobrir-lhe as feições tão maternas.
Abri o portão principal como quem abria
Um cofre que resguardava valores incomensuráveis.
As vozes de todos os dias estavam reinauguradas.
Deitei-me no colo de minha mãe como se quisesse
Realizar a proeza de ser gerado de novo.
Suas mãos sobre os meus cabelos pareciam devolver-me
A mim mesmo.
Mãos com poder de sutura existencial....
Era como se o gesto possuísse voz, capaz de me dizer:
dorme minha filha, porque enquanto você dormir
Eu lhe farei de novo. Dorme minha filha, dorme....

Padre Fábio de Melo

Sumário

"Alegrai-vos sempre no Senhor. Seja conhecida de todos os homens a vossa bondade. Não vos inquieteis com nada! Em todas as circunstâncias apresentai a Deus vossas preocupações, mediante a oração, as súplicas e a ação de graças. E a paz de Deus que excede toda a inteligência, haverá de guardar vossos corações e vossos pensamentos, em Cristo Jesus."

Filipenses 4:4

Capítulo 1

O PAPEL DO DIABETE NAS ALTERAÇÕES DA MATRIZ EXTRACELULAR E SEU IMPACTO NA CONTINÊNCIA URINÁRIA

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RESUMO

A prevalência de Incontinência Urinária (IU) em gestantes diabéticas é significantemente elevada e permanece alta 2 anos após o parto cesárea. Esses achados evidenciam que o diabete na gestação é fator de risco para o desenvolvimento de IU nas mulheres que não tiveram a influência da via de parto. Os estudos desse binômio diabete e IU na gestação são escassos na literatura. A incontinência pode ser a consequência mais comum da hiperglicemia comparada a outras complicações, assim, identificar os fatores de risco para o desenvolvimento da IU em diabéticas é o maior objetivo na prevenção desta condição tão comum. Pesquisas recentes apontam que não apenas o músculo mas também a matriz extracelular (MEC) uretral desempenham importante papel no mecanismo da continência urinária. Os trabalhos translacionais do nosso grupo de pesquisa evidenciaram que o diabete na prenhez de ratas lesa a matriz extracelular e o músculo estriado uretral, o que pode explicar a alta prevalência de IU e disfunção do assoalho pélvico em mulheres com *diabete mellitus* gestacional. O diabete exerce efeito na expressão, organização e modificação nos componentes da MEC em diversos órgãos e a remodelação do tecido e a fibrose parecem ser uma consequência direta do diabete. Assim, entendendo o impacto de fatores de risco modificáveis como o diabete, implica que o uso de estratégias preventivas pode reduzir as taxas de IU e melhorar a qualidade de vida das mulheres, especialmente na gestação e no pós-parto.

Palavras-chave: diabete, incontinência urinária, matriz extracelular, uretra

1. INTRODUÇÃO

A prevalência de 50,8% de Incontinência Urinária (IU) em gestantes diabéticas é significantemente elevada e permanece alta 2 anos após o parto cesárea (1). Esses achados evidenciam que o diabete na gestação é fator de risco para o desenvolvimento de IU nas mulheres que não tiveram a influência da via de parto.

A junção de diabete e IU é aspecto relevante em termos de saúde pública e de economia em saúde. O mundo vive hoje uma epidemia de diabete e obesidade, e isto tem influência direta nos custos de saúde (2). A IU é também uma epidemia silenciosa que atinge 200 milhões de pessoas em todo mundo e gerou um custo direto nos Estados Unidos de aproximadamente US$ 20 bilhões de dólares no final do século passado (3, 4).

Os estudos do binômio diabete e IU na gestação são escassos na literatura. Nosso grupo de Pesquisa iniciou a pesquisa translacional em ratas prenhes com diabete de intensidade grave e moderada com Marini *et al.* (2011) e Piculo *et al.* (2013), que evidenciaram que o diabete e prenhez em ratas danificam o músculo estriado e a matriz extracelular uretral (5, 6).

O primeiro trabalho com diabete de intensidade grave (glicemia maior que 300mg/dL), demonstrou adelgaçamento e atrofia do músculo estriado uretral associado à desorganização e rompimento das fibras. A análise imunoistoquímica evidenciou perda da localização anatômica normal das fibras com aumento na proporção de fibras lentas (5).

No segundo trabalho, com o intuito de mimetizar o diabete com intensidade glicêmica moderada (glicemia entre 120-300mg/dL), verificamos que além das alterações musculares, foram encontradas mudanças significativas na matriz extracelular como fibrose e na ultraestrutura como acúmulo de mitocôndrias, aumento de gotas de lipídios e acúmulo de grânulos de glicogênio no grupo diabético moderado prenhe (6).

As alterações encontradas nos dois trabalhos do nosso grupo de pesquisa podem explicar a alta prevalência de IU e disfunção do assoalho pélvico em mulheres com DMG. Como o

músculo esquelético é o órgão alvo principal do metabolismo de glicose, o entendimento dos mecanismos básicos envolvendo a miopatia diabética tem enorme importância para o desenvolvimento de estratégias terapêuticas para melhorar a qualidade de vida das gestantes diabéticas e reduzir os custos da assistência à saúde. Os achados desses dois modelos evidenciam que o diabete na prenhez de ratas lesa o músculo uretral de forma similar.

Pesquisas recentes apontam que não apenas o músculo mas também a matriz extracelular (MEC) uretral desempenham importante papel no mecanismo da continência urinária (7, 8).

Assim, verificando que a literatura ainda é controversa na discussão do comportamento da MEC na gênese da IU e nos efeitos do diabete e gestação, o objetivo desta revisão foi estudar o papel da matriz extracelular frente à incontinência urinária, a gestação e o diabete.

2. CARACTERIZAÇÃO FISIOLÓGICA DA MATRZ EXTRACELULAR

Além do componente muscular estriado e liso, a parede da uretra é composta por tecido conjuntivo especializado para cada região, tais como na região da lâmina própria urotelial, entre as células do tecido muscular liso e entre as células musculares estriadas do esfíncter uretral. É de extrema importância conhecer a composição, distribuição e organização dos componentes da MEC destas diferentes regiões do tecido uretral e as modificações existentes na condição de IU, gestação e diabete visando contribuir para melhor entendimento das alterações uretrais frente a estas condições.

O tecido conjuntivo é constituído por fibroblastos e uma complexa rede de macromoléculas denominada matriz extracelular. A MEC contém colágeno, elastina, fibronectina, laminina e proteoglicanos (9-11) que, em conjunto, conferem propriedades bioquímicas e biofísicas específicas aos diferentes tecidos (12). É importante ressaltar que a MEC constitui uma rede tridimensional ao redor de todas as células, órgãos e tecidos do corpo. Em geral, a MEC forma um filtro biofísico para proteção e nutrição bem como meio para

facilitar a resposta imune e regeneração tecidual (13). Corresponde a 20% do nosso peso corporal e trata-se de um ambiente dinâmico formado por componentes fibrilares e um gel viscoso constituído de macromoléculas altamente hidratadas (14).

Os componentes estruturais da MEC, como colágeno e fibras elásticas, proporcionam rigidez mecânica e flexibilidade ao tecido, servindo também como substrato para adesão e migração celular (15). Além disso, as proteoglicanas (PGs) e glicosaminoglicanas (GAGs) da MEC atuam cooperativamente aos componentes fibrilares para regular os processos de proliferação, adesão, migração, diferenciação e apoptose celular (16). As moléculas da MEC também formam a membrana basal (que separa o epitélio ou endotélio do estroma), constituída principalmente por colágeno IV, lamininas, nidogem e perlecan, que auxilia na manutenção da arquitetura e integridade tecidual do trato genito-urinário (17-19).

O sistema colágeno consiste de diferentes tipos de fibras colágenas, fibras reticulares, colágenos de ancoragem e colágenos associados a fibrilas. Já o sistema elástico apresenta fibras finas e ramificadas, formando uma rede bastante irregular (13). O colágeno é o maior componente da MEC; existem 28 tipos de colágeno descritos em todo o corpo, mas os mais abundantes no assoalho pélvico são os tipos I e III (20). O colágeno tipo I é o maior colágeno de formação fibrilar. Ele polimeriza nas fibras maiores e é o determinante primário de força tensional. O colágeno tipo III predomina nos vasos sanguíneos e está relacionado com flexibilidade e distensibilidade dos tecidos (21).

O microambiente celular influencia o comportamento e sobrevivência das células. A MEC exerce não somente funções mecânicas e estruturais, mas também se associa a fatores solúveis e receptores transmembrana para coordenar espacialmente os processos de sinalização celular (19). A capacidade das células em reconhecer química, mecânica e topograficamente as características e componentes da MEC permite a estas células responder de modo coerente aos estímulos do microambiente ao seu redor (22). Essa interação célula-matriz regula propriedades

celulares fundamentais, tais como a diferenciação e a pluripotencialidade celular (23). Esses aspectos do comportamento celular são essenciais para o desenvolvimento, maturação e homeostase dos órgãos (24). Assim, a identificação e a quantificação dos componentes distintos da MEC, sua dinâmica espacial e temporal representam passos importantes na compreensão do papel da MEC na saúde e na doença (19). A literatura evidencia que alterações na dinâmica da MEC estão associadas à fibrose (22), inflamação crônica (25) e ao câncer (26).

O arranjo, o grau de deposição e a manutenção das fibras colágenas também dependem de outros componentes da MEC, principalmente de proteoglicanas e glicosaminoglicanos (27). As PGs são componentes abundantes na MEC, consistindo de um esqueleto protéico no qual cadeias de GAGs e oligossacarídeos estão ligados (28). As PGs estão envolvidos na manutenção das propriedades estruturais, mecânicas e biológicas da MEC (29), na regulação da proliferação e mobilidade celular e nas interações célula-célula e célula-matriz (30).

As GAGs são heteropolissacarídeos, formados por unidades dissacarídicas repetitivas compostas de uma hexosamina e um açúcar não aminado, com exceção o queratam sulfato, que apresenta uma galactose substituindo o ácido urônico. As GAGs mais comuns são condroitin sulfato (CS), dermatan sulfato (DS), heparan sulfato (HS), queratan sulfato (QS), heparina (H) e ácido hialurônico (AH - único que não está ligado covalentemente ao esqueleto protéico das PGs e não apresenta grupamento sulfato em sua cadeia) (31). Devido seu caráter polianiônico, as GAGs são capazes de interagir com uma variedade de componentes presentes tanto na MEC quanto na superfície celular. Assim, esses polímeros podem inibir ou regular a passagem de outras moléculas através da membrana basal, controlar o acesso de macromoléculas (fatores de crescimento e hormônios) à superfície celular (32), afetar o crescimento, migração, adesão e a diferenciação celular (33).

A eficiência do colágeno é influenciada pelas metaloproteinases de matriz (MMPs). As MMPs atuam no processo de remodelação dos componentes da matriz extracelular e, portanto,

auxiliam na manutenção da homeostasia tecidual. Elas são endopeptidases dependentes de metais, principalmente zinco e cálcio. Possuem a capacidade de degradar os componentes da MEC, como colágeno, elastina, laminina, fibronectina e proteoglicanos (34). São sintetizadas na forma de um precursor latente, que necessita sofrer um processamento proteolítico (remoção do pró-peptídeo) para se tornar ativo (35). Atualmente, são descritos cerca de 23 tipos de MMPs, divididas em 6 grupos com base na especificidade do substrato, similaridade sequencial e organização dos domínios. Dentre os tipos: 1) colagenases intersticiais (MMP-1, 8 e 13); 2) gelatinases A e B (MMP-2 e 9, respectivamente); 3) estromelisinas (MMP-3, 10 e 11); 4) matrilisinas (MMP-7 e 26); 5) metaloproteinases de membrana (MT-1, 2, 3, 4, 5 e 6-MMP ou MMP-14 a 17, 24 e 25, respectivamente) e 6) MMPs que não se enquadram nessas definições (MMP-19, 23 e 28) (24, 36). Enquanto as MMPs são secretadas, as MT-MMPs possuem um domínio transmembrana que as ancora à membrana plasmática, onde participam da cascata de ativação de outras MMPs ou atuam diretamente sobre um substrato (37).

A atividade e a produção das MMPs são controladas por inibidores endógenos conhecidos como inibidores teciduais de metaloproteinases ou TIMPs (38-40). Quatro TIMPs foram identificados em mamíferos: TIMP-1, TIMP-2 e TIMP-4, que são proteínas secretadas. O TIMP-3 encontra-se ancorado na MEC. A expressão dos TIMPs é regulada durante o desenvolvimento e remodelação tecidual e o mecanismo de inibição parece ocorrer devido à ligação do domínio N-terminal do TIMP ao domínio catalítico da MMP (41). O desequilíbrio entre a ação das MMPs e dos TIMPs pode resultar em diversas condições patológicas, como por exemplo artrite reumatóide, doenças cardiovasculares (42) e na progressão dos tumores de próstata, cólon, melanomas, mama e urotélio (43-48).

3. MATRIZ EXTRACELULAR NA CONTINÊNCIA E INCONTINÊNCIA URINÁRIA

A IU de esforço resulta tipicamente da disfunção do mecanismo de fechamento do esfíncter uretral e/ou dos tecidos circundantes que fazem a uretra não resistir ao fluxo de urina nos períodos de aumento da pressão intra-abdominal (49). Diversas pesquisas indicam que a MEC uretral desempenha papel crítico na patogênese da IU (7, 8).

A literatura descreve diferenças na composição e na quantidade de colágeno em mulheres com e sem IU. Alguns autores relacionaram IU com diminuição na quantidade de colágeno. Rechberger *et al.* (50) observaram uma redução significativa de 20% no colágeno total na fáscia pubocervical em mulheres com IU. Liapis *et al.* (51, 52) também verificaram diminuição no colágeno tipo I e III na fáscia vaginal e ligamento uterosacrais de mulheres com IU e prolapso. Em 2007, Trabucco *et al.* (53) e Song *et al.* (54) sugeriram um remodelamento do tecido conjuntivo na região periuretral de pacientes com IU, com diminuição e distribuição anormal das fibras colágenas, resultando em uma matriz mais flexível. Todos esses autores concluíram que o colágeno tem importante papel na manutenção da continência urinária, porém o mecanismo envolvido ainda não está claro.

Por outro lado, muitos autores relacionam a IU com aumento na quantidade de colágeno periuretral em mulheres (55-57). Em 1998, Falconer *et al.* verificaram, em biópsia uretral, que a concentração de colágeno e o diâmetro das fibrilas colágenas eram 30% maior no grupo de mulheres incontinentes e concluíram que a IU está associada a mudanças no metabolismo do colágeno, levando ao aumento na sua concentração e no tamanho de suas fibras. Estas alterações podem resultar em uma MEC mais rígida e prejudicar sua função mecânica (57). Fitzgerald *et al.* (2000) também encontraram diferenças morfológicas na ultraestrutura uretral em biópsias de mulheres incontinentes. Houve presença de padrão degenerativo, desorganização nas fibras colágenas e aumento na quantidade de proteoglicanos na superfície do colágeno em algumas pacientes com IU (56). Devido à diversidade nos métodos de detecção, tipo de biópsia e

avaliação dos desfechos, um consenso ainda não foi alcançado. Além disso, as amostras ainda são heterogênias e em pequeno número para permitir conclusões satisfatórias (40).

Com relação as GAGs, Feldner *et al.* (2006) verificaram que mulheres incontinentes apresentaram maior quantidade total de GAGs sulfatadas e dermatan sulfato no tecido periuretral. Os autores hipotetizaram que a IU pode estar relacionada com mudanças bioquímicas nos componentes da matriz extracelular do tecido conjuntivo periuretral e que as GAGs contribuem para as propriedades físicas, já que estão envolvidas na resistência e elasticidade tecidual. Devido à interação com o colágeno, sugere-se então que as GAGs representam papel fundamental na complacência e resistência de suporte às estruturas pélvicas (58).

Apesar dos estudos quantitativos de colágeno e do arranjo fibrilar possuírem resultados divergentes na literatura, a disfunção do assoalho pélvico pode ser um problema decorrente da diminuição na síntese ou do aumento da quebra de colágeno (40).

Em estudos experimentais, Mitrano *et al.* (18) avaliaram os efeitos da prenhez no perfil das GAGs na bexiga e na uretra de ratas adultas. Em todos os grupos, o dermatan sulfato predominou seguido pelo heparan e ácido hialurônico. A presença de condroitin não foi observada. O tecido uretral apresentou aumento nas GAGs totais no 20° dia de prenhez. Este aumento de GAGs totais poderia mudar a complacência tecidual, gerando uma matriz mais resistente às forças de compressão. As diferenças nas concentrações e síntese de GAGs encontradas na bexiga e na uretra sugerem que o tecido conjuntivo pode estar relacionado às alterações no trato urogenital.

Takano *et al.* (27) avaliaram o efeito do trauma simulado do parto, parto vaginal e cesárea nas GAGs nas uretras de ratas. Após quatro dias de experimento, maior quantidade de GAGs foi encontrada nas fêmeas prenhes comparado à quantidade encontrada em outros grupos. Com 12 semanas, maior quantidade de GAGs foi encontrada no grupo com parto cesárea

seguido do parto simulado. Concluíram que há mudanças nas GAGs sulfatadas nas uretras de ratas durante a prenhez e após o trauma de parto simulado e que estas mudanças podem afetar as propriedades mecânicas do tecido e, consequentemente, prejudicar os mecanismos de continência urinária. Estas mesmas análises foram realizadas por Ruano *et al.* no tecido vaginal de ratas. Os resultados mostraram menor quantidade de GAGs e dermatan sulfato durante a prenhez e após o parto natural com quatro dias de experimento. Os autores acreditam que os efeitos isolados da prenhez, sem os eventos de trauma, cesárea ou parto vaginal, induzem diminuição dos GAGs. Estas alterações podem estar relacionadas com diminuição nos níveis hormonais pós-parto mais do que no trauma vaginal. As diferentes concentrações de GAGs no tecido vaginal sugerem que o tecido conjuntivo pode estar relacionado com desordens no trato urogenital como prolapso e IU (59).

Sabe-se que as alterações hormonais decorrentes da gestação são responsáveis por grande parte das disfunções no trato urinário inferior (60). Estudos verificaram que o hormônio relaxina, membro da família da insulina de hormônios peptídicos e produzido exclusivamente pelo corpo lúteo (61), aumenta a atividade das metaloproteinases (MMP) da matriz no trato urogenital em mulheres (62) e pode diminuir a formação de fibrose (63). Este efeito anti-fibrótico pode prejudicar a habilidade de auto-reparo do assoalho pélvico no período pós-parto e, consequentemente, resultar em disfunção do mesmo. As MMPs desempenham importante papel no remodelação tecidual e estão relacionadas com vários processos fisiológicos, como quebra de colágeno e reparo tecidual (64). De fato, elevados níveis de MMPs tem sido encontrados em mulheres com prolapso genital (65) e IU (66, 67).

Há controvérsias sobre a etiologia de IU em mulheres fora da gestação e do parto. Alguns autores sugerem que fatores genéticos podem aumentar a probabilidade de uma mulher desenvolver IU e disfunção muscular do assoalho pélvico (40, 68). Che *et al*. identificaram que genes relevantes da MEC são encontrados no tecido vaginal de mulheres com IU quando

comparados aos de mulheres continentes. Os genes de expressão diferencial foram TGFβ-3, laminina, colágeno tipo IV e proteínas relacionadas à função dos miócitos. O TGFβ-3 está envolvido com estimulação na produção da MEC e com aumento na produção de colágeno tipo I e III, os principais componentes responsáveis pela força tênsil de tecidos ligamentosos (68).

Connell *et al.* demonstraram um aumento de duas vezes na expressão de MMP-2 em mulheres com prolapso pélvico comparada a de mulheres controle normais (69). Outros estudos também confirmaram o aumento na atividade da pro-MMP-e e da atividade da MMP-2 por imunoistoquímica no mesmo grupo de mulheres (70, 71). Chen *et al.* encontraram aumento de 80% nos níveis de expressão da MMP-1 na parede vaginal anterior de biópsias de mulheres com IU e com prolapso (72). Estes investigadores também notaram diminuição concomitante na expressão do TIMP-1 nas mesmas pacientes (72) bem como em outro estudo envolvendo tecido periuretral de mulheres com IU (73).

4. MATRIZ EXTRACELULAR NO DIABETE E INCONTINÊNCIA URINÁRIA

A relação entre DM e IU tem sido observada em diversos estudos epidemiológicos (74-76), mas os mecanismos pelos quais DM leva à IU ainda são escassos. A taxa de IU entre mulheres com diabete é de 50 a 200%, mais elevada do que entre mulheres com níveis normais de glicose (77-79). Há evidência que a IU é mais prevalente no diabete do que outras complicações comuns associadas a esta síndrome, como retinopatia, nefropatia e neuropatia (80, 81). Gharaee-Kermani *et al*.(2013), sugeriram que a obesidade, o DM tipo 2, a fibrose no trato urinário inferior e a perda urinária estão intrinsecamente e biologicamente ligados (82).

O diabete exerce efeito na expressão, organização e modificação nos componentes da MEC em diversos órgãos (83-85). Estas alterações vem sendo descritas em humanos, por exemplo, no músculo estriado esquelético (85) e na artéria renal (86) e em ratos no tendão da

cauda, (87), decídua (88), pele (89), tendão de Aquiles (90), fígado (91), próstata (92) e uretra (93, 94).

A remodelação do tecido e a fibrose parecem ser uma consequência direta do diabete. Uma vez que a fibrose interrompe a função normal de órgãos em indivíduos diabéticos, os miofibroblastos, como um dos moduladores primários da produção e volume da MEC, são alvo terapêutico potencial para a prevenção da remodelação da matriz patológica. Na fibrose tubulointersticial observada na nefropatia diabética, o aumento do número de miofibroblastos têm sido associado com a deposição excessiva de MEC (84).

Berria *et al.* observaram aumento significativo de colágeno tipos I e III em biópsia muscular do vasto lateral de pacientes com DM tipo 2, concluindo que mudanças na composição da MEC são características do músculo insulino-resistente. É possível que estas alterações na matriz do músculo esquelético sejam uma resposta à inflamação crônica, que leva à patologia mitocondrial e resulta em acúmulo de lipídio intracelular e resistência à insulina. Porém, este cenário é apenas especulativo e necessita de mais estudos para explicar este mecanismo (85).

Estudos experimentais evidenciam aumento na deposição de colágeno no trato urinário inferior em animais diabéticos. Ratas após 6-8 semanas com diabete induzido por *streptozotocin* (glicemia maior que 300mg/dL), após distensão vaginal, apresentaram aumento na deposição de colágeno entre as fibras do músculo estriado uretral. Os autores concluíram que o acúmulo de espécies reativas de oxigênio e os produtos finais de glicação avançada podem contribuir para as causas miopáticas e neuropáticas das disfunções do trato urinário inferior e que o diabete está associado com incontinência mais severa e com atraso na recuperação das injúrias nos mecanismos da continência urinária (93).

Rodrigues *et al.* analisaram as alterações estruturais da MEC uretral em ratos machos Wistar com diabete induzido por aloxana. Foi verificado que a atrofia muscular e fibrose uretral ocorreram após 44 semanas e que este processo foi mais severo no grupo diabético do que nos

animais não-diabéticos. Assim, as mudanças estruturais na matriz extracelular uretral são relevantes e devem ser consideradas nos estudos do trato urinário (94).

Ahmed *et al.* encontraram deficiência na síntese de colágeno I e III nos tendões de ratos diabéticos, sugerindo produção desregulada das metaloproteinases. A expressão protéica de MMP-13 foi maior nos tendões de ratos diabéticos, entretanto, essa diferença não foi observada na análise de expressão correspondente. Inversamente, a expressão gênica de MMP-3 foi menor nos tendões de ratos diabéticos (95). A discrepância entre os resultados da expressão gênica e protéica pode ser explicado pela regulação da transcrição ou pós-tradução influenciada, potencialmente, pelos níveis elevados de glicose no diabete (96).

5. CONCLUSÃO

Uma das principais causas de IU de esforço são as alterações presentes no músculo e matriz extracelular uretral. O diabete é um dos principais causadores destas complicações, mas ainda há controvérsias nas consequências da gestação neste mecanismo. Os dados sugerem que a incontinência pode ser a consequência mais comum da hiperglicemia comparada a outras complicações. Identificar os fatores de risco para o desenvolvimento da IU em diabéticas é o maior objetivo na prevenção desta condição tão comum. Enquanto a predisposição genética, paridade e idade tem um importante papel no desenvolvimento da IU de esforço, o impacto de fatores de risco modificáveis como o DM, implica que o uso de estratégias preventivas pode reduzir as taxas de IU e melhorar a qualidade de vida das mulheres, especialmente na gestação e no pós-parto.

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"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 às coisas."

Apocalipse 21:1

Capítulo 2

DISRUPTION OF COLLAGEN AND GLYCOSAMINOGLYCANS STATUS IN THE URETHRAL TISSUE OF PREGNANT RATS WITH *STREPTOZOTOCIN* INDUCED SEVERE DIABETES

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ABSTRACT

A positive relationship between gestational diabetes and urinary incontinence was established by our group. The aim of this study was to analyze the distribution and quantification of the key structural extracellular matrix components, including total collagen, collagen I and III, collagen I/III ratio and sulfated glycosaminoglycans, in urethra of severe STZ-induced diabetic pregnant rats. **Methods:** One hundred and twenty female Wistar rats were distributed in four experimental groups: virgin, pregnant, diabetic and diabetic pregnant. In adult life, diabetes was induced in rats by *streptozotocin* injection administered intravenously at 40 mg/kg to produce a permanent and severe diabetic state (blood glucose level >300 mg/dL). At day 21 of the experiment, the rats were lethally anesthetized and the urethra and vagina were extracted as a unit. Urethral and vaginal sections were cut and analyzed by a) histochemical staining for extracellular matrix and muscle structural components and morphometric analysis**,** b) immunohistochemistry to identify collagen I and III and Keratan sulfate, and c) Protein extraction and western blotting analysis for Collagen I, Collagen III and Keratan sulfate. **Results:** The total striated muscle is not only decreased but also this striated muscle is involved by more connective tissue characterized by an increase in the relative ratio of the collagen I/III and a decrease in total GAGs and Keratan sulfate. **Conclusion:** The importance of this study is that it provides the first line of experimental evidence in support of a metabolic relationship between the elevated glycemic levels and urethral dysfunction in diabetic pregnant rats.

Keywords: diabetes, extracellular matrix, pregnancy, rats, urethral striated muscle.

1. INTRODUCTION

The impact of Gestational *Diabetes mellitus* (GDM) on postpartum urinary incontinence have rarely been investigated. Our group (Barbosa *et al*., 2011) and Chuang *et al*., (2012) separately established a positive relationship between GDM and urinary incontinence (UI) (1, 2). It had been reported that damages on the urethral striated and smooth muscle are the key components in this pathogenesis (3, 4).

In a previous study we described that severe diabetes induced by *streptozotocin* (STZ) in pregnant rats caused morphological changes in the muscle mass and fast and slow fiber profiles. The urethral striated muscle was found to be thin, atrophic, disorganized, and disrupted. They were associated with colocalization of fast and slow fibers and the loss of the predominance of the expression of the fast fibers. The results of this translational study suggest that UI may be attributed, in part, to the changes in urethral striated muscle of diabetic pregnant women (5). Not only severe diabetes impacts the urethral muscles in rats but also the mild STZ-induced diabetes in pregnancy. There are structural, ultrastructural, pathological and immunohistochemical alterations that allow us to confirm that the diabetic myopathy in mild diabetic pregnant rats are also involved in the pathogenesis of UI (6).

Dorschner *et al*.,(2001) emphasize the significance of the sphincterally arranged muscles at the vesico-urethral junction and in the urethral wall (7). In urodynamic studies, most stress-incontinent women show a reduced intraurethral pressure. These findings imply a deficient mechanism of urethral closure in stress-incontinent women. It is likely that the pelvic connective tissue is vital for maintaining urinary continence. The hypotheses of the authors suggest that a functionally changed pelvic connective tissue affects the mechanism of urethral closure (8, 9).

The female urethra is surrounded by a fibrous-muscular system of connective tissue. Researchers have indicated that the urethral extracellular matrix (ECM) may also play a critical role in UI beside the urethral striated muscle (10-13). ECM is composed by several key elements

such as collagen, elastic fibrils, proteoglycans, and glycosaminoglycans (GAGs). They are organized like a hammock, which is important to morphologic diversity and tissue functions, and it is considered an important factor for genitourinary tract supportive structures (14).

The mechanical properties of connective tissue are determined by the structure of the individual matrix molecules in the tissue, their related interactions and their overall proportions (15). Alterations in ECM components have been reported in women with urinary incontinence as well as in periurethral tissues of incontinente women with increase in collagen fibers (16, 17) and sulfate GAGs (18).

Extracellular matrix is essential for tissue remodeling, and many of its components provide a physical structure support to the surrounding cells. More importantly, proteins have a key role in regulating survival, motility, proliferation, and morphology of normal cells and contribute to a variety of cellular functions, including organ development, wound healing, and metabolism (14, 19).

There are approximately 21 collagen subtypes; however, the urethra, bladder and vagina are composed predominantly of collagen types I and III (11). Collagen I forms the largest and strongest fibers and Collagen III forms smaller fibers. Even small variations in the ratios of fibrillar collagen subtypes can significantly alter the tensile strength of a tissue. For this reason, the relative ratio of the collagen I to III is used as an indicator of tensile strength (20).

In diabetic individuals, ECM components are frequently affected in various tissues, suggesting that changes in the expression and distribution of these molecules are common characteristics of this pathology (21). Therefore, modification of ECM proteins by glycation in diabetes may have a severe impact on cellular function (22). Some studies have examined the relationship between ECM components, especially collagen, and urinary incontinence (23), diabetes (22) and pregnancy (24), but always separately.

Considering that GDM increase UI incidence and pelvic floor muscle dysfunction; the structure and distribution of extracellular matrix components and their components that determine the biochemical properties of the tissue, the aim of this study was to analyze the distribution and quantification of the key structural extracellular matrix components, including total collagen, collagen I and III, collagen I/III ratio and sulfated glycosaminoglycans, in urethra of STZ-induced severe diabetes in pregnant rats. The difficulty in obtaining tissues from human female studies on UI mechanism in diabetic women are lacking so this animal model provides us an opportunity to quantify the components of urethral ECM and the possible therapy methods. Our hypothesis is that the deep study of extracellular matrix give the opportunity to better understand the mechanisms related to UI incidence and pelvic floor muscle dysfunction in diabetic women and provide a perspective to introduce new therapeutic approaches.

2. MATERIALS AND METHODS

All of the experimental protocols were approved by our Institutional Animal Care and Use Committee (process number 828-2010). Female Wistar rats at 90 days of life were used. The animals were randomly allocated into four groups: the virgin group (N=30), the pregnant group (N=30), the diabetic group (N=30) and the diabetic pregnant group (N=30). Diabetes was induced in rats by *streptozotocin* injection (STZ; SIGMA Chemical Company, St. Louis, MO, USA). The STZ was administered intravenously at 40 mg/kg to produce a permanent and severe diabetic state. Blood samples were taken 72 h after STZ injection to confirm diabetes (blood glucose level >300 mg/dL) (25). 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. The experimental sequence was showed in Figure 1.

In the diabetic pregnant group, diabetes was induced by STZ (intravenously at 40 mg/kg) seven days prior to mating. The female rats were mated overnight with non-diabetic male rats.

The morning when sperm was found in the vaginal smear was designated as the gestational day 0.

The rats were killed on day 21 of the experimental by i.p. Thiopentax® injection at 80 mg/kg. The offspring were removed, weighed and lethally anesthetized with sodium thiopental (3% Thiopentax®) and the maternal urethrovaginal tissues were harvested (cross-section of the mid-urethra and anterior vagina). The investigators controlled the longitudinal axis (proximal to distal) of the urethra by marking with a permanent ink pen to identify the distal urethra. All the analyses were performed in the same points along the urethral longitudinal axis: mid-urethra region, where the striated muscle layer becomes denser.

Histological examination, immunohistochemical stain, and morphometric analysis

A portion of the samples (N=10 samples/group) was immersed in neutral buffered formalin containing 4% formaldehyde for a period of 4h and embedded in paraffin. Sections of 4µm thickness were cut in the mid-urethra using a rotor microtome and stained with Masson's trichrome and Picrosirius red for histological examination and morphological analyses. Specimen cross sections were examined by normal and polarized light and photographed. Morphometric analysis was performed with Image Pro Plus 7.0 image analysis software (Media Cybernetics, Ins. USA) at Case Western Reserve University (USA).

For immunohistochemistry (N=10 samples/group), samples were frozen in liquid nitrogen and kept at -80°C for cryostat sectioning (6 µm thick). Sections were incubated with antibodies directed towards Collagen I (1:500; ab90395; Abcam™), Collagen III (1:500; ab6310; Abcam™), Keratan Sulfate (1:300; clone 373E1, Kamiya Biomedical Company).

Protein extraction and western blotting analysis for Collagen I, Collagen III and Keratan sulfate

The urethra frozen samples (N=5 samples/group) were mechanically homogenized in 50 mM Tris–HCl buffer pH 7.5, 0.25% Triton X-100 and EDTA by means of a Polytron homogenizer (Kinematica, Lucerne, Switzerland) for 30 s at 4 °C. Following centrifugation of the homogenate, the protein was extracted from the supernatant and was quantified as described by Bradford (1976). Equal amounts of protein (75 μg) from the urethra frozen samples were heated at 95 °C for 5 minutes in the sample-loading buffer and were then subjected to SDS–PAGE under reducing conditions and were transferred to nitrocellulose membranes (Sigma Chemical Co., St. Louis, MO).

The blots were blocked with 3% bovine serum albumin in TBST (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 hour and probed overnight with the primary antibody, anti-Collagen I (1:500; ab90395; Abcam^{TM}), anti-Collagen III (1:200; ab6310; Abcam^{TM}), anti-Keratan Sulfate (1:500; clone 373E1, Kamiya Biomedical Company). Goat anti-β-actin antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) served as loading control. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies, the blots were detected by means of chemiluminescence (Immun-Star^{TM} HRP Chemiluminescent Kit, BIO-RAD). Protein expression was expressed as integrated optical density (IOD). The Collagen I, Collagen III and Keratan sulphate expression was normalized to the β-actin values. The analysis was performed with ImageJ 1.48a software (Wayne Hasband, National Institute of Health). Normalized data are expressed as the means±SD.

Quantification and characterization of sulfated glycosaminoglycans

After washing in PBS (Phosphate-Buffered Saline - 5 mM phosphate buffer, 0.15 M NaCl and 50 mM EDTA), total urethra (n = 5) were immersed into acetone for 12h and dried in a histological oven (37 °C) for 12 h. GAGs were extracted with papain solution (40 mg/g tissue) in 100 mM sodium phosphate buffer, pH 6.5, containing 40 mM EDTA and 80 mM β-

mercaptoethanol for 24 h at 50 °C. After precipitation using 90% TCA (trichloroacetic acid) for 10 min at 5 °C, the samples were centrifuged and submitted to precipitation with methanol during 12 h at 5 °C. The precipitate was resuspended in water and used for the measurement of GAGs (26). Before electrophoresis, 5 μg of each sample was treated with DNAse (10 mg/mL) in 20 mM Tris–HCl buffer, pH 7.4 for 30 min at 37 °C. Then, the sulfated glycosaminoglycans (GAGs) chondroitin (CS), dermatan (DS) and heparan sulfate (HS) were separated by electrophoresis in agarose gel (0.5%) in 0.05 M propylene diamine (27) at 0.1 mA for 45 min. The agarose gels were fixed in cetavlon and stained with 0.2% TB. The gels were washed with a solution containing 50% ethanol and 1% acetic acid for observation of bands. The identification of GAGs was confirmed by digestion with chondroitinases B and AC. The analysis was performed with ImageJ 1.48a software (Wayne Hasband, National Institute of Health). Data are expressed as the means±SD (pixels).

Statistical analysis

Results are expressed as means ± SD. Comparisons of measurements among the virgin, pregnant, diabetic and diabetic pregnant groups were performed individually by ANOVA followed by Test of Tukey Multiple Comparison for variables with normal distribution. Dunnett's test was applied for comparison of the study group “diabetic pregnant” versus the other three “controls” groups (virgin, pregnant and diabetic). $P < 0.05$ was considered significant. All analyzes were performed using the SAS software for Windows v.9.2.

3. RESULTS

To analyze the pathological changes in the urethral extracellular matrix of diabetic pregnant rats, several methods were applied. We first performed histological and morphometric analysis with Masson's Tricome and Picrosirius red. Polarized light examinations of urethra

stained with Picrosirius red showed no apparent differences in structural organization of collagen fibers between the groups (Figure 2). Striated muscle of the urethra of virgin and pregnant groups is compact and nearly circumferentially with little collagen infiltration. In diabetic groups, mild focal disruption of striated muscle fibers was found with increased deposition of collagen fibers around the striated muscle.

Morphometric analysis revealed that no differences were found in the urethral connective tissue area/total area ratio and urethral connective tissue in smooth area/smooth muscle area between the groups. Urethral connective tissue in striated area/striated muscle area and urethral connective tissue in striated area/ urethral connective tissue in smooth area ratios were significantly greater in Diabetic Pregnant group than Pregnant group. All mean values and standard errors of these morphologic variables are listed in Table 1.

To localize the distribution of collagen I and collagen III we performed immunohistochemical staining. The results showed that for Collagen I and III, the staining was homogeneously distributed in the extracellular matrix in four groups (Figures 3,4).

With regard to the average of Collagen I and Collagen III expression by Western blot analysis there was no significant difference in between the groups (Figures 2,3), however the ratio of collagen I/III in the urethra was increased in the Diabetic Pregnant group in relation to Virgin group (Figure 5).

The results of total sulfated GAG quantification analysis (mg/g tissue) are showed in Figure 6. The figure showed that there is a significantly reduced GAG content in the Diabetic Pregnant group. Further analysis of the GAG by densitometry showed that the amount of dermatan sulfate was predominant in all groups without significantly difference between them. Heparan sulfate was significantly lower in Diabetic compared to Virgin group (Figure 6). Two GAGs were not identified caused by low-molecular-weight: chondroitin sulfate (CS) and keratan sulfate (KS).

The results of Keratan sulfate analysis by immunoistochemistry and Western blot showed a significant reduction in the Diabetic Pregnant group (Figure 7).

4. DISCUSSION

Female urinary incontinence is a common symptom that disables many Gestational Diabetic women two years after cesarean delivery, but its etiology is still unclear (1). In a previous translational study we found that urethral striated muscle in diabetic rats were thin, atrophic, disorganized, disrupted and associated with loss of the predominance of the fast fibers, confirming that diabetic myopathy is involved in the pathogenesis of UI (5, 6). These studies confirmed that a reduction in muscle content may negatively affect structural and functional integrity of the urethra (5, 6, 13, 28).

The present study showed in severe diabetic pregnant rats that the two major components of urethral tissue (connective tissue and striated muscle) are altered. The total striated muscle is not only decreased but also this striated muscle is involved by more connective tissue characterized by an increase in the relative ratio of the collagen I/III and a decrease in total GAGs and Keratan sulfate. These changes in collagen concentration might thus also affect the contractile properties of the muscle (29).

The increase of collagen type I/ III ratio is opposite to that found in the pathology of urethral fibromuscular system related to parturition-induced the stress urinary incontinence (13). Connective tissue quality is mainly determined by the amount and ratio of synthesized and deposited collagens type I and type III. Mature type I collagen, found in dense bundles in connective tissue is responsible for tensile strength. In contrast, type III collagen fibers are thinner in diameter and are regarded as the immature collagen predominantly found in early wound healing (30, 31). For this reason, the relative ratio of collagen I/III is used as an indicator of tensile strength. A reduced ratio of type I to type III collagen is known to change the geometrical arrangement and diameter of collagen fibrils and to decrease the amount of cross-

linking, with reduced mechanical stability of connective tissue (20, 31). Our results showing an increased ratio of type I to III collagen confirms the importance of biological approach to the understanding of the pathogenesis of UI in diabetic patients. The higher collagen Type I/III ratio increase the tensile strength of ECM opposite to the results found in the pathology of urethral fibromuscular system related to parturition-induced stress urinary incontinence (13).

It is well known that collagen fibrils in striated muscle play an important role in intercellular transmission of active force although the literature is controversial. Changes of the amount and distribution of different types of collagen can influence the biomechanical properties of the bladder and urethra. It has been suggested that the ratio of collagen types I and III is an important factor related to bladder and urethral dysfunction (32, 33).

Our results highlighted the difference between ECM from urinary incontinence related to parturition and those induced by diabetes and pregnancy in rats. ECM related to parturition leads a decrease in collagen I/III ratio indicating a soft tissue (13), although our results demonstrated that ECM related to STZ-induced diabetes leads an increase of collagen I/III ratio, suggests a more rigid structure, supportive collagen around the urethra in favor of a stiffer urethral tissue. This can impair the biomechanical properties of the tissue making the closure of the urethra more difficult.

Pelvic support relies on a functional connective tissue ECM (10). Following different lines of research, studies have investigated alterations in the pelvic floor connective tissue in UI. Some authors correlated UI with decreased collagen fibers (15, 17, 34-36), while others correlated with an increased of collagen (16, 37). Thus, there is no well-accepted conclusion of whether UI correlates with collagen (13). Research into glycosaminoglycans is still in its beginning stages, and to our knowledge there are still no data on GAGs composition in urethral tissue in severe STZ-induced diabetic pregnant rat model.

Extracellular components, in diabetic individuals, are frequently affected in various tissues, suggesting that changes in the expression and distribution of these molecules are common characteristics of this pathology (21). Gharaee-Kermani *et al*.(2013), suggest that obesity, Type 2 *Diabetes mellitus*, lower urinary tract fibrosis and urinary voiding dysfunction are inextricably and biologically linked (38). Diabetes-associated alterations in collagen (tissue stiffness) have been described in human studies in arterial kidney (39) and skeletal muscles (40), and in experimental studies in rats with tail tendon (41), decidua (42), skin (43), Achilles tendons (44), liver (45), prostate (46), and urethra (28, 32).

After vaginal distension, diabetic animals, showed an obvious thinning and atrophy of the urethro-vaginal septum and a focal increased deposition of yellow collagen fibers around the striated muscle of the urethra. The authors supported a hypothesis that diabetes is associated with more severe incontinence, and a delayed recovery from injuries to the continence mechanisms in female rats undergoing vaginal distension as a surrogate of childbirth (28). These findings were confirmed by Ebbesen *et al*. (2013) that found an association between diabetes and reduced odds of remission from UI (47).

Patients with diabetes are at greater risk to suffer the morbidity of various musculoskeletal disorders (48). Studies indicated that elevated glucose levels impair collagen production and also intensify the presence of advanced glycation end products (AGEs), resulting in abnormal ultrastructure of collagen fibrils (41, 49). A common complication of type 2 diabetes is delayed or defective tissue healing, resulting from inadequate production of growth factors, compromised angiogenesis, and impaired formation of a collagen matrix (50). In this pathological state, non-enzymatic glycation may also influence collagen fibrillogenesis by affecting the interaction between collagens and proteoglycans (51).

Extracellular matrix proteins are susceptible to AGE modification because of their slow turnover rate. The formation of intermolecular and intramolecular crosslinks with collagen as a

result of the glycation process leads to structural alterations, increased stiffness and resistance to proteolytic digestion (52). ECM remodeling in insulin-resistant skeletal muscle is of potential significance for muscle metabolism, and increased abundance of types I and III collagen are found in skeletal muscle biopsies in patients with Diabetes Type 2 (40).

The role of dysregulation of matrix metalloproteinases (MMPs) which degrade the extracellular matrix, and the endogenous tissue inhibitors of metalloproteinases (TIMPs), which regulate the activity of the metalloproteinases (53) have also been implicated in the pathophysiology of several diabetic complications, however, the exact roles of MMP and TIMP within the context of DM remain controversial (54). The expression of glomerular MMP-2 was found to be reduced, and TIMP-2 to be increased, in the kidney tissues of early diabetic patients (55) and lower levels of MMP-2 and MMP-9 in subjects with type 2 diabetes were noted (56).

All these findings have implications on therapeutic. The traditional approach could be conservative namely physiotherapy, although the reason for poor success lies in their collagen content, for despite increase their pelvic floor strength, the stiffer connective tissue connecting these muscles to the urethra could not transmit the forces of muscular contraction and on the other side surgery may not be beneficial to this group too. Evidently, control of the blood glucose level is a good strategy in the treatment of diabetic urinary incontinence. It is often difficult to maintain blood glucose at a level that can prevent UI. Therefore it is necessary to use the processes leading to urethral dysfunction as a framework for design of novel therapeutic targets (29, 33).

The results of our study should be interpreted by awareness of the following limitations: rats are quadrupeds; they have tails with associated musculature and STZ-induced diabetes does not reproduce human disease (28). We selected rat because it is a small animal and our group have great experience with diabetes and pregnancy investigation of the role of such mechanisms in our model requires additional investigation.

5. CONCLUSIONS

STZ-induced diabetes and pregnancy result in marked remodeling of urethral tissue, which included reduction in striated muscle and GAG content and increase in collagen I/III ratio. This different organization of collagen status and profile of glycosaminoglicans found in our urethral samples suggest that this pathology of urethral fibromuscular system could be related to diabetic-induced UI. The higher collagen Type I/III ratio increase the tensile strength of ECM opposite to the results found in the pathology of urethral fibromuscular system related to parturition-induced stress urinary incontinence (13). The importance of this study is that it provides the first line of experimental evidence in support of a metabolic relationship between the elevated glycemic levels and urethral dysfunction in diabetic pregnant rats. To address these questions large cohort studies are required with analysis of collagen type I/III ratio as a means to identify diabetic pregnant women at high risk for UI.

6.ACKNOWLEDGEMENTS

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7. COMPETING INTERESTS

The authors declare that there are no competing interests.

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EXPERIMENTAL SEQUENCE

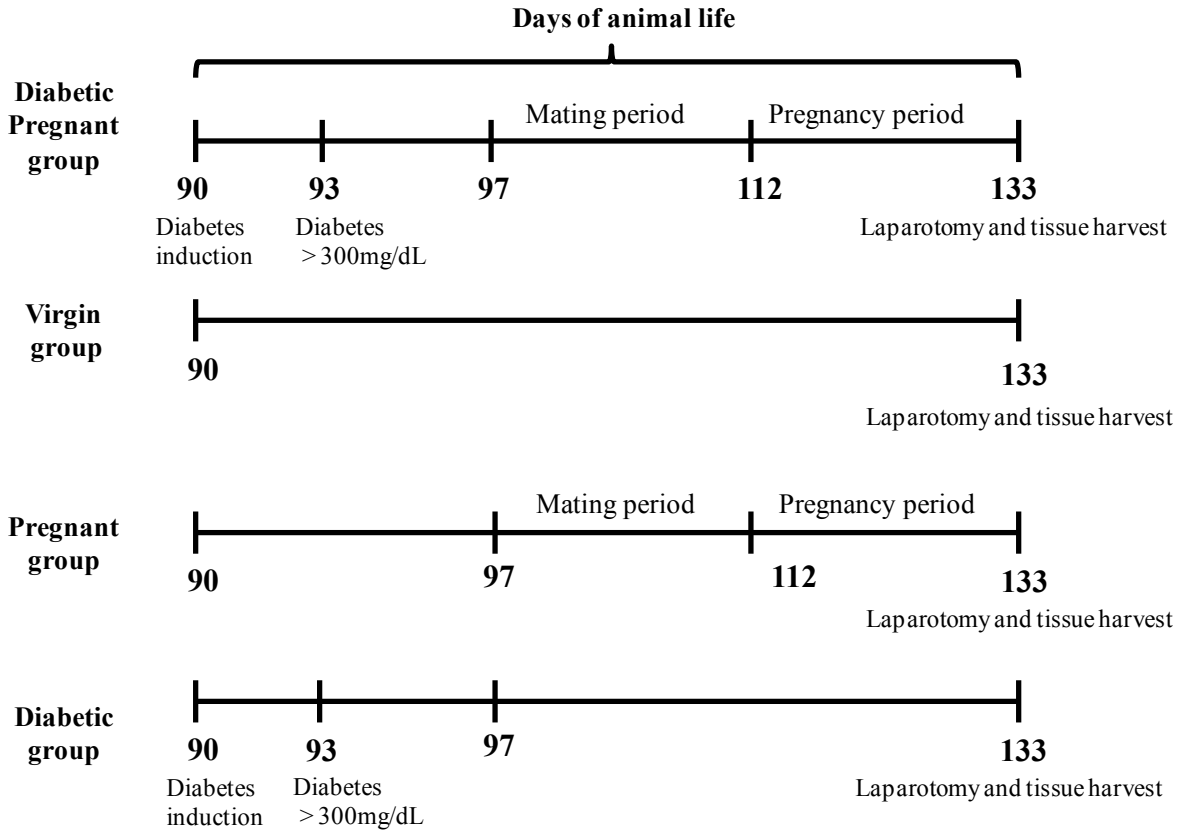

Figure 1. Experimental sequence of the groups.

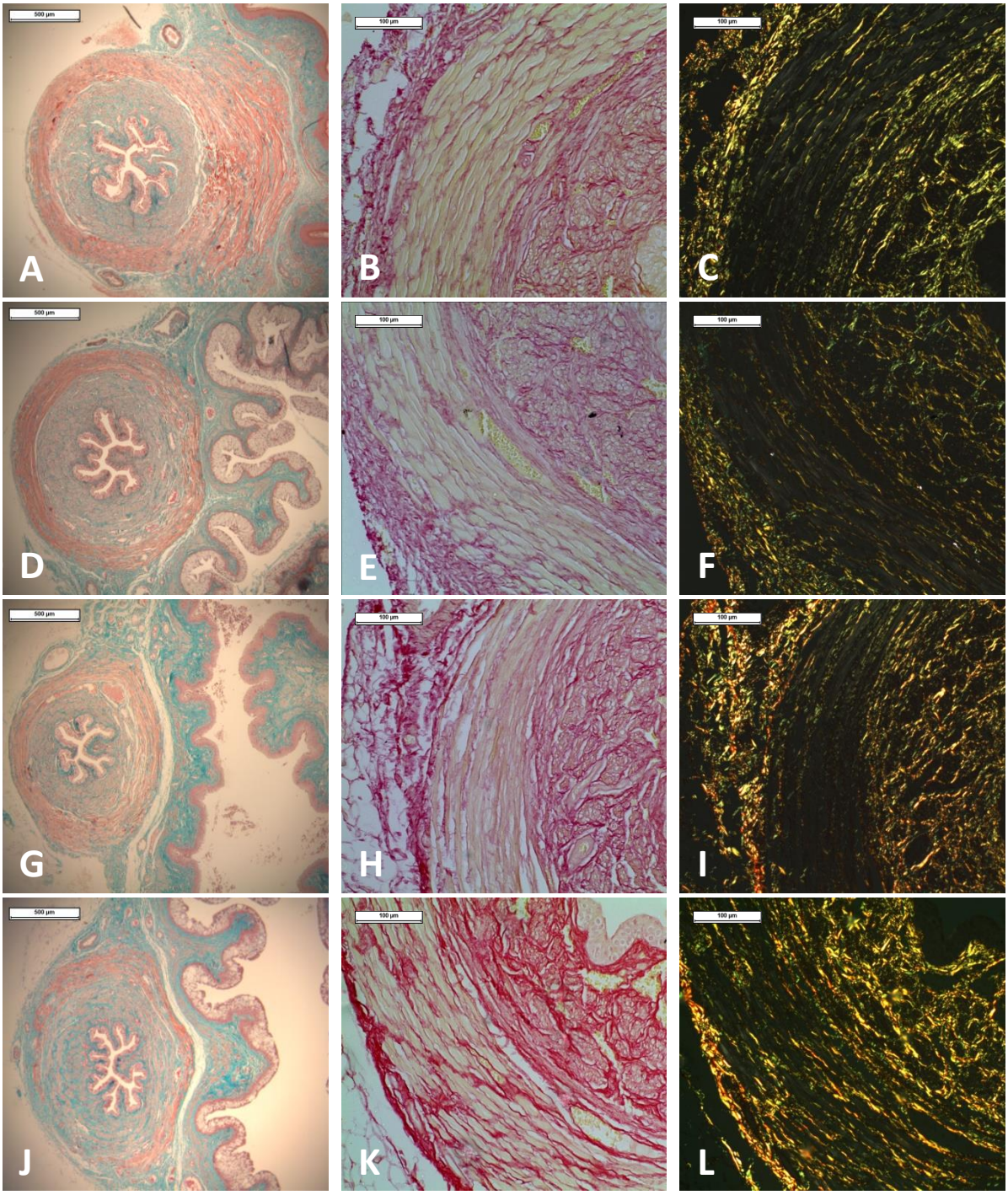

Figure 2. Transverse section of urethra stained by Masson's Tricome (A,D,G,J), Picrosirius red observed under normal (B, E, H, K) and polarized light (C, F, I, L). Virgin group (A, B, C), Pregnant group (D, E, F), Diabetic group (G, H, I) and Diabetic Pregnant group (J, K, L).

Table 1. Morphometric ratios of fibromuscular system in diabetic pregnant group compared to the virgin, pregnant and diabetic rats.

	VIRGIN a	PREGNANT b	DIABETIC c	DIABETIC PREGNANT
Urethral striated muscle area/total area	0.37 ± 0.05	0.33 ± 0.04	0.31 ± 0.06	0.28 ± 0.08**a**
Urethral connective tissue area/total area	0.33 ± 0.08	0.33 ± 0.05	0.34 ± 0.12	0.39 ± 0.11
Urethral connective tissue in striated area/striated muscle area	0.48 ± 0.17	0.40 ± 0.07	0.49 ± 0.17	0.71 ± 0.37**b**
Urethral connective tissue in smooth area/smooth muscle area	0.70 ± 0.26	0.76 ± 0.18	0.89 ± 0.30	0.65 ±0.25
Urethral connective tissue in striated area/ urethral connective tissue in smooth area	1.50 ± 0.40	0.94 ± 0.31	1.10 ±.42	1.65 ± 0.82 **b**

The results are expressed in mm^2. Values are means ± SD. (Dunnett test)
a *P < 0.05 - Significantly different from virgin group*
b *P < 0.05 - Significantly different from pregnant group*
c *P < 0.05 - Significantly different from diabetic group*

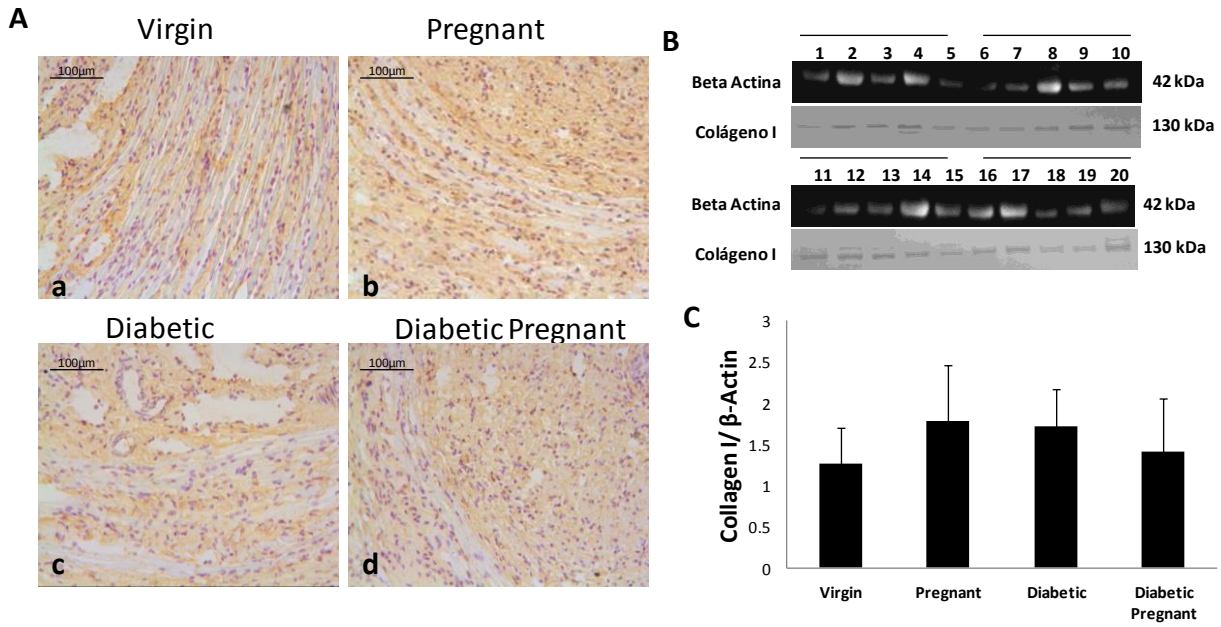

Figure 3. Assessment of extracellular matrix in the urethra. **A** Representative photographs of cross-sections of mid-urethra with immunoistoquemistry of Colagen I in four groups: virgin (a), pregnant (b), diabetic (c) and Diabetic pregnant (d). **B** Western blot results indicated there was no significant difference in the average of Collagen I expression between the groups Virgin (1-5), Diabetic (6-10), Pregnant (11-15) and Diabetic Pregnant (16-20). **C** Data are presented as the relative density of Collagen I compared with that of β-Actin. Each bar depicts the mean value ± SD.

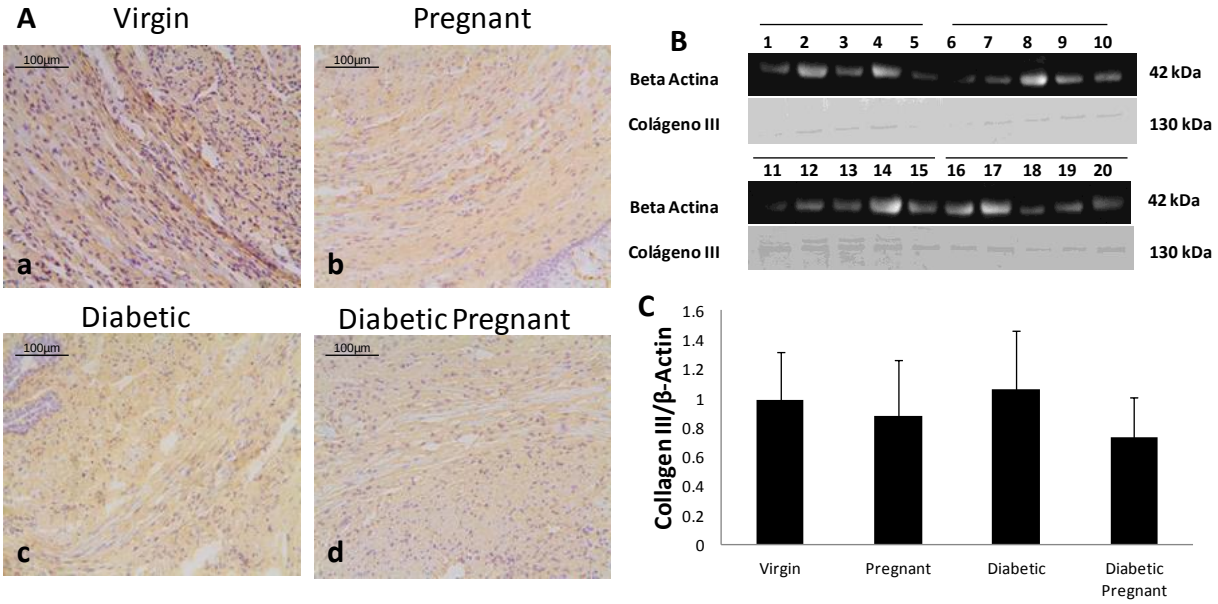

Figure 4. Assessment of extracellular matrix in the urethra. **A** Representative photographs of cross-sections of mid-urethra with immunoistoquemistry of Colagen III in four groups: virgin (a), pregnant (b), diabetic (c) and Diabetic pregnant (d). **B** Western blot results indicated there was no significant difference in the average of Collagen III expression between the groups Virgin (1-5), Diabetic (6-10), Pregnant (11-15) and Diabetic Pregnant (16-20). **C** Data are presented as the relative density of Collagen III compared with that of β-Actin. Each bar depicts the mean value ± SD

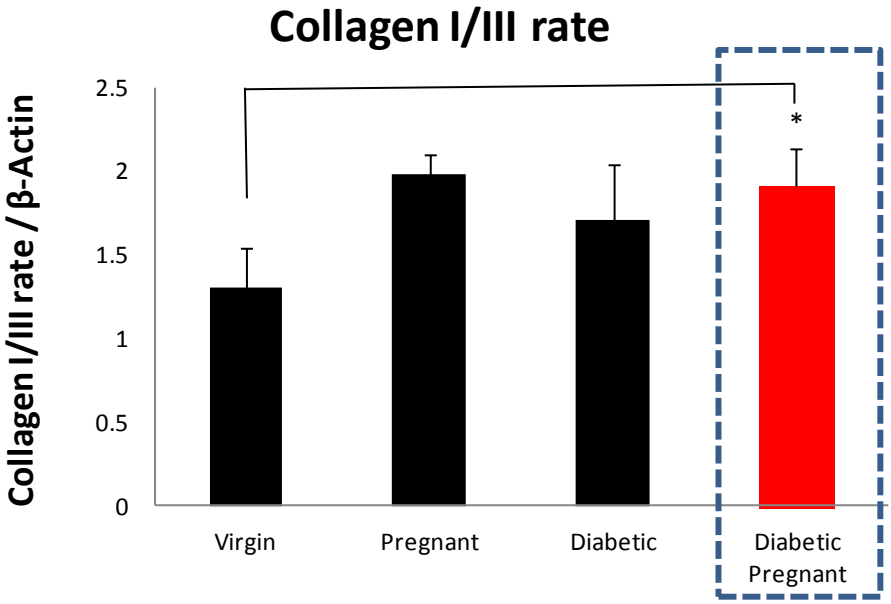

Figure 5. Relative density of Collagen I and III compared with that of β-Actin and Collagen I/III ratio. Diabetic Pregnant group showed higher Collagen I/III ratio compared to Virgin group.

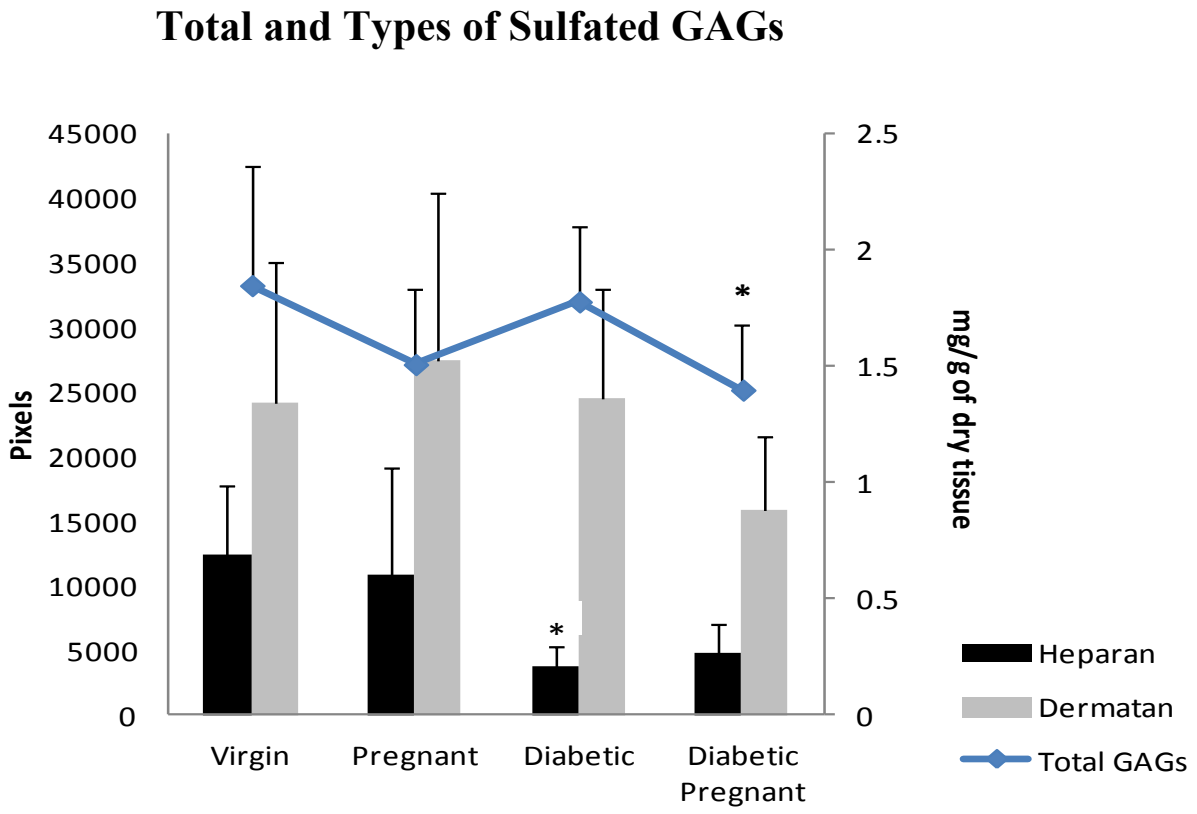

Figure 6. Total concentrations of sulfated glycosaminoglycans in four groups. Diabetic Pregnant group were significantly lower than Virgin group. Values are expressed as milligrams of glycosaminoglycans per gram of dry tissue ± standard deviation (right vertical axis). Comparison of Dermatan sulfate and Heparan sulfate in four groups. Values are expressed in pixels ± standard deviation (left vertical axis).

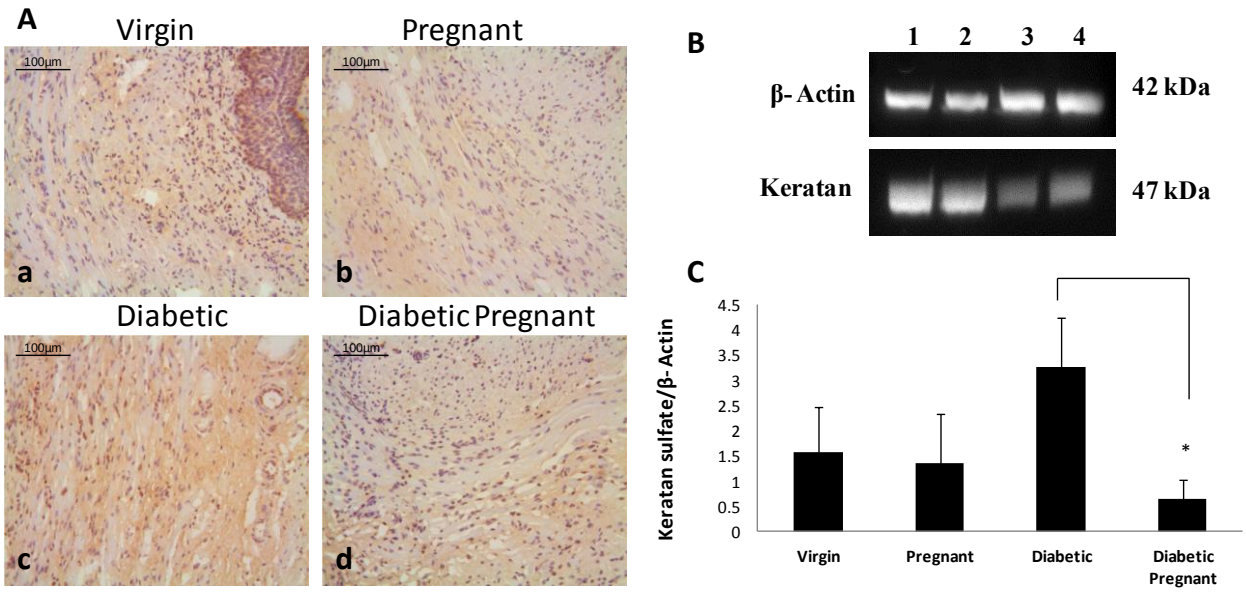

Figure 7. Assessment of extracellular matrix in the urethra. **A** Representative photographs of cross-sections of mid-urethra with immunoistoquemistry of Keratan Sulfate in four groups: virgin (a), pregnant (b), diabetic (c) and Diabetic pregnant (d). **B** Decrease in Keratan Sulfate expression in Diabetic Pregnant group rats compared with Diabetic group by western blot: 1 (Virgin), 2 (Diabetic), 3 (Pregnant) and 4 (Diabetic Pregnant). **C** Data are presented as the relative density of Keratan Sulfate compared with that of β-Actin. Each bar depicts the mean value ± SD.

"Every road is a slippery slope
But there is always a hand that you can hold on to
Looking deeper through the telescope
You can see that your home is inside of you"
Jason Mraz

Capítulo 3

EFFECTS OF SHORT-TERM SEVERE AND LONG-TERM MILD STZ-INDUCED DIABETES IN URETHRAL TISSUE OF FEMALE RATS

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ABSTRACT

Introduction and Hypothesis: *Diabetes mellitus* affects multiple organs including urinary system either in diabetic or in prediabetic stages. Recent evidence strongly suggests that urinary incontinence is a common complication among women with diabetes. In additional to these studies, we and other investigators showed that diabetes induced alterations of urethral tissue in rats. Our hypothesis was that the intensity and number of alterations on urethral striated muscle and extracellular matrix of short-term severe diabetes were higher compared to long-term mild diabetes in rats. **Methods:** For the induction of mild diabetes (blood glucose between 120-300 mg/dL), female newborns received *streptozotocin* (100 mg/kg body weight, sc route) and to induction of short-term severe diabetes (blood glucose level >300 mg/d) adult animals received *streptozotocin* at 40 mg/kg, iv route. The rats were killed on day 133 of the experimental by i.p. Thiopentax® injection at 80 mg/kg and the urethrovaginal tissues were harvested. Morphometric, pathological, immunohistochemical to fast and slow fibers, and ultrastructural analysis were conducted. **Results:** In long-term mild diabetes were found collagen deposition, severe fibrosis, lipid droplets and numerous subsarcolemmal and intermyofibrillar mitochondria. The effect of STZ-induced short-term severe diabetes and long-term mild diabetes on urethral striated muscle and ECM of female rats resulted in diabetic myopathy in both models which included decrease number of fast fibers and loss of specific localization of fibers type I and type II. **Conclusion:** Long-term mild diabetes amplifies the alterations found in urethral tissue with severe fibrosis and has important implications for the monitoring and treatment strategies implemented in patient with *diabetes mellitus*.

Key-words: diabetes, rats, urethra, extracellular matrix, striated muscle.

1. INTRODUCTION

Diabetes mellitus (DM) a chronic disease characterized by hyperglycemia, affects multiple organs including urinary system either in diabetic or in prediabetic stages (1). Recent evidence strongly suggests that urinary incontinence (UI) is a common complication, from 50 to 200% more common among women with DM2 compared to normal glucose levels (2-4). In additional to these studies, we and other investigators showed that diabetes induced alterations of urethral tissue in rats (5-8). The risk factors involved in UI development are many, however, the association with diabetes is of great interest at present. These data underline the importance of understanding the abnormalities on urethral tissue of DM2 in order to offer better target and more effective treatment or even prevention of the disease.

Skeletal muscle is one of the major organs responsible for whole body insulin resistance under insulin stimulated conditions (9) and muscle biopsies in diabetes research have revealed a variety of cellular abnormalities in patients with DM2 and prediabetes (10). More recently, the application of global approach such as proteomics and gene expression profiling on skeletal muscle biopsies has pointed to alterations in mitochondrial oxidative phosphorylation in DM2. These novel insights will inevitably cause a renewed interest in studying skeletal muscle. Of course this approach comes together with a discussion of the advantages and limitations of the methods in diabetes research.

In a translational study it is possible to analyze both factors together: duration and intensity of glycemic levels. To obtain severe diabetes models, the *streptozotocin* (STZ) is administrated in adult rats (day 90), and by the other side to obtain mild diabetes, the STZ is administrated on the first day of life (day 0) (11).

This temporal history of diabetes-induced and the intensity of glycemic levels are both aspects not well studied. It is well known the time-dependent repercussions of heart diseases as well as temporal diabetes-induced diuresis as etiology for remodeling of urinary bladder in rat

(8, 12). The effects of short-term severe hyperglycemia in urethral tissue are unanswered or have not been addressed in sufficient detail. The time-dependent changes in urethral tissue in rats could confirm that abnormalities in prediabetic subjects are more likely due to environmental factors.

Our hypothesis was that the intensity and number of alterations on urethral striated muscle and extracellular matrix (ECM) of short-term severe diabetes were higher compared to long-term mild diabetes. The scope of this paper is to provide a comparative and critical account of two different STZ diabetes models for the assessment of urethral tissue. We illustrate the basic technical aspects of the methodologies, referring the readers to the original publications for the details of protocol.

2. MATERIALS AND METHODS

All of the experimental protocols were approved by our Institutional Animal Care and Use Committee (process number 828-2010). Female and male Wistar rats were housed in a certified animal care facility and food and water were provided *ad libitum* and maintained under controlled conditions (temperature 22 ± 2°C, humidity 55 ± 5% and 12h light/dark cycle). To evaluate short-term severe diabetes and long-term mild diabetes, two parallels studies were conducted. All animals were killed at 133 days of life. The experimental sequence is showed in Figure 1.

First study: Long-term mild diabetes

Parental non-diabetic female rats were mated with non-diabetic males to obtain female newborns. For the induction of mild diabetes (blood glucose between 120-300 mg/dL), female newborns received *streptozotocin* (STZ - 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 (13). 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. Newborn rats remained with their mothers until day 21 of life (weaning period). Glucose tolerance test (GTT) was performed at day 75 of life according to Campos *et al*. (14) to assess the development of altered glucose metabolism and used as a criterion for include the rats in the certain group. For rats that presented glycemia higher than 140 mg/dL in more than two measures during GTT continued in the experiment. Other female newborns received only citrate buffer and these animals were considered as non-diabetic and included in this study as a control group.

Second study: Short-term severe diabetes

Short-term severe diabetes was induced (90 days of age) in rats by *streptozotocin* injection (STZ; SIGMA Chemical Company, St. Louis, MO, USA). The STZ was administered intravenously at 40 mg/kg to produce a permanent and severe diabetic state. Blood samples were taken 72 h after STZ injection to confirm 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 control group.

Tissue Harvest

The rats were killed on day 133 of the experimental by i.p. Thiopentax® injection at 80 mg/kg and the urethrovaginal tissues were harvested (cross section of the midurethra and anterior vagina). Investigators controlled the longitudinal axis (proximal to distal) of the urethra by marking with a permanent ink pen to identify the distal urethra. All analyses were performed at the same points along the urethral longitudinal axis, midurethra region, where the striated muscle layer becomes denser.

Morphometric, pathological, immunohistochemical, and ultrastructural analysis

A portion of the samples (N=10 samples/group) was immersed in neutral-buffered formalin containing 4% formaldehyde for a period of 4h and embedded in paraffin. Sections of 4 μm thickness were cut in the mid-urethra using a rotor microtome and stained with Masson's trichrome for morphological analyses. Specimen cross sections were examined by light microscopy and photographed. Morphometric analysis was performed with Image Pro Plus 7.0 image analysis software (Media Cybernetics, Ins. USA) at Case Western Reserve University (USA). This study measures the proportion of urethral tissue that is of each of 5 types: striated muscle, smooth muscle, collagen, urothelium, and blood vessel. The data is normalized so that the proportions add to 100% for each sample.

For pathological analysis, which was done by a pathology specialist at Case Western Reserve University (USA), forty Masson's trichrome stained photo/file slides from the urethra of 4 groups were analyzed for the grade of fibrosis (4X magnification). The fibrosis was considered:

- Minimal if it was present at the muscularis propria.
- Minimal to moderate if it is minimal but had an increased intensity.
- Moderate if it was penetrating through the muscularis propria.
- Severe if it was moderate in grade but had an increased intensity.

The pathological analysis was qualitative and allowed no comparison between groups, but it could be confirmed by morphometric analysis that quantitatively allowed to measuring the specific collagen area.

Additional samples (10 samples/group) were frozen in liquid nitrogen and kept at -80 °C for midurethra sectioning in a cryostat (6 μm thick) for immunohistochemical analysis of fast and slow fibers. Antibodies WB-MHCf Novocastra (1:120) and WB-MHCs Novocastra (1:180) were used.

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. In the first moment, the presence of each type of fiber throughout circumference of the layer; the thickness of muscle fiber layers and the degree to which the layers maintained a normal anatomic localization were investigated.

Other samples (3 samples/group) were immersed in the fixative solution containing 0.05% ruthenium red for 3h before postfixation in osmium tetraoxide. After staining, the usual procedures for transmission electron microscopy were performed for ultrastructural analysis of urethral striated muscle.

Data analysis (morphometric, pathological, immunoistochemical and ultrastructural) in mild diabetes group was published by Piculo *et al.,* in 2013 (6).

Statistical analysis

Results are expressed as means ± SD. Comparisons of measurements among the groups were performed individually by ANOVA followed by Test of Tukey's Multiple Comparison for variables with normal distribution. Poisson distribution was performed when data presented no homogeneous distribution. $P < 0.05$ was considered significant. All analyzes were performed using the SAS software for Windows v.9.2.

3. RESULTS

General physical characteristics (body weight and blood glucose levels) observed in this study were compatible with mild and severe diabetes. This fact was confirmed because the long-term mild diabetes group presented glycemic values ranging from 120 to 300 mg/dL and short-term severe diabetes group presented glycemic values >300mg/dL (data not shown). Both non-diabetic groups showed similar results and was analyzed together as a control group. Table 1

summarizes the morphological changes of urethral tissue in control, long-term severe diabetes and short-term mild diabetes groups.

The plot (Figure 2) shows how the percentage of each tissue type changes among the groups. For example, the upper left shows the average percent for each tissue type in the control group. The upper right shows the same for the long-term mild diabetes group. And the upper middle shows the difference between them. So, collagen significantly increased from 30.05 (height of middle bar in upper left plot) to 40.00 (upper right) for an increase of 9.95 (upper middle). All plots are on the same scale so comparisons of bar heights can be made directly.

The short-term severe diabetes group showed significantly lower striated muscle area and higher blood vessel area compared to control group. Compared to long-term mild diabetes, the striated muscle and total collagen significantly decreased and blood vessel increased in short-term severe diabetes group (Figure 2).

The **pathological analysis** is showed in Figure 3. The figure showed that severe fibrosis only occurred in long-term mild diabetes. These data reinforce the potentially deleterious effects of temporality more than hyperglicemic levels.

The **immunohistochemical** staining in the control group 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. A thin, inner circular layer of slow striated muscle fibers was observed with individual fibers being small and thin. The image shows different localization patterns for each type of fibers, with fast fibers being outermost and slow fibers innermost. In short and long-term diabetes, immunohistochemical staining revealed a loss of specific localization for each type of fiber, with changes of fast to slow fibers and a decrease the number of fast fibers (Figure 4, 5). These results were insufficient to detect influence of different levels of glycemia as well as temporality

In the **ultrastructural analysis**, the urethral striated muscle in the control group showed well-organized myofibrils forming intact sarcomeres with morphological characteristics related to different muscle types, with no signs of change. Abundant intermyofibrillar mitochondria and rare lipid droplets were observed. There was no increase in the interstitial connective tissue and the collagen was normally distributed. Glycogen granules normally dispersed were observed (Figure 6).

The long-term mild diabetes group caused an increased interstitial collagen, lipid droplets and numerous subsarcolemmal and intermyofibrillar mitochondria in the striated muscle cells. The glycogen granules were dispersed in larger quantities. In the short-term severe diabetes group centrally located myonuclei presence and sarcoplasmic reticulum sparse T tubes was noted (Figure 6).

4. DISCUSSION

The heterogeneity of clinical symptoms of diabetes may result from a variety of factors including the duration and the type of diabetes (8). Therefore, to identify the effect of temporality diabetes as well as the severity of glucose levels on urethral tissue, two different models have been put forth to evaluate which is more important the high blood glucose levels or the duration of the hyperglycemia insult. This design would have allowed us to compare the severity and natural long-term history of diabetes.

The details and pathophysiological mechanisms of such changes are not well understood (8). Morphological studies from both models have revealed a variety of cellular abnormalities. The short-term severe diabetes undergoes decrease in striated muscle, increased capillary density and centrally myonuclei presence that indicates muscle injury (16).

Comparing short-term severe diabetes with long-term mild diabetes in the present study, we demonstrated that both models present similar immunohistochemistry patterns characterized

by loss of specific localization for each type of fiber, changes of fast to slow fibers and a decrease in the number of fast fibers. These results suggest that hyperglycemia is responsible for this fact, independently of the level.

Diabetes is associated with accumulation of reactive oxygen species, and tissue ischemia which can interactively or independently contribute to the myopathy causes of skeletal muscle dysfunctions (17, 18). It is well established that the muscle fibers are able of altering their physiological and biochemical properties according to stimulus which they are submitted, reflecting either on amount or on the type of muscle proteins (19). These changes are often associated with altered glucose metabolism, diabetes and obesity.

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 (20). Striated fibers are the dominant muscle components of the mid-urethra (21) and have been classified into two major groups, type I (slow twitch) and type II (fast-twitch) fibers, based on the presence of myosin heavy chain (MHC) isoforms. Slow-twitch type-I muscle fibers are rich in mitochondria, exhibit a high oxidative capacity, and are resistant to fatigue. Conversely, fast-twitch type II muscle fibers have robust glycolytic metabolism and fatigue easily (22). The roles of each fiber type in striated sphincter contraction are controversial and depend on the species studied and the method used to determine the fast and slow fiber types (23). As both models present the same characterization, it allows us to define that the diabetic myopathy depends from the hyperglycemia, independent from its duration.

Researchers have indicated that the urethral extracellular matrix (ECM) may also play a critical role in UI beside the urethral striated muscle (24-27). Collagen is the major constituent of the ECM in urethra. Our findings of collagen deposition, severe fibrosis, lipid droplets and numerous subsarcolemmal and intermyofibrillar mitochondria in long-term mild diabetes group are consistent with reports from clinical evidence that women with prediabetes are also at higher

risk for UI. In the National Health and Nutrition Examination Survey 2001-2002, women with impaired fasting glucose had an increased prevalence of UI similar to that in women with diabetes (33,4% and 35,4%, respectively) and significantly higher than in women with normal fasting glucose (16,8%) (2). The increase of collagen and severe fibrosis might thus also affect the contractile properties of the urethra (8). Our and others previous studies demonstrated increased in deposition of collagen fibers around the striated muscle of the urethra of diabetic animals compared with those of control animals (5-7).

Berria *et al*., concluded after human insulin-resistant muscle biopsies that there was increased abundance of types I and III collagen, and these data suggest that, just like in lipid-induced experimental insulin resistance, naturally occurring insulin resistance is associated with increased collagen expression in skeletal muscle and an altered ECM. It is possible that an alteration in the ECM in skeletal muscle, perhaps in response to chronic inflammation, could lead to mitochondrial pathology that might ultimately result in an accumulation of intramyocellular lipid and insulin resistance (28).

The ultrastructural analysis of the striated muscle cells in long-term mild diabetic rats showed an increased interstitial collagen, glycogen granules, lipid droplets, and numerous mitochondria, the same found by Piculo et *al*. (6). Lipid droplets are not membrane bound, and their number and size may vary considerably among different muscle types. They are frequently associated with mitochondria and sometimes completely encircled by them. Experimental enzyme deficiencies in mitochondrial energy metabolism induced the accumulation of giant mitochondria and numerous lipid droplets. This has led to the suggestion that mitochondria may use lipids as a source of energy for muscular contraction. The increased number of lipid droplets in the diabetic rats indicates that lipids may be an important energy source for striated muscle (25), and lipid oversupply can result in a dramatic increase in the expression of ECM genes in

skeletal muscle from healthy subjects. These genes included several collagen genes fibronectin, proteoglycans, and connective tissue growth factor (29).

Vascular complications are associated with diabetes (30) which agree with significant increase in blood vessels in severe diabetes group. Hyperglycemia in humans or animal models of diabetes is associated with impaired mitochondrial activity predominantly in vascular tissues resulting in mutations within mitochondrial DNA, reactive oxygen species production, apoptosis, and endothelial dysfunction (31).

These findings were unexpected and provided remarkable evidence. The low intense changes in short-term severe diabetes showed that poor glycemic control is less aggressive for urethral tissue in rats than glucose tolerance deviated slightly from the normal during a long period of time. These findings may motivate the need to identify mild hyperglycemia as a risk factor that contributes to the development of UI. The relationship between DM2 and UI has been observed in several epidemiological studies (32-34) although the influence of the lower-threshold criteria for glycemic levels is not well recognized. It may reasonably be inferred that subclinical hyperglycemia during a long period predisposes to silent lesions in urethral tissue that may impair the function of continent mechanisms.

Identify risk factors for development of UI is a major goal in prevention of this common and distressing condition. The substantial impact of modifiable risk factor such as low threshold values for diabetes diagnosis could be an interesting preventive strategy to reduce UI and improve women's quality of life. A translational study using these results should be a priority (35).

5. CONCLUSION

In conclusion, the effect of STZ-induced short-term severe diabetes and long-term mild diabetes on urethral striated muscle and ECM of female rats resulted in diabetic myopathy in both models which included decrease number of fast fibers and loss of specific localization of fibers type I and type II. Time-dependent diabetes insult increases the collagen amount, severe fibrosis, lipid droplets and mitochondria. These data suggest that time-dependent is responsible for stronger alterations in muscle and collagen compared to severity of glycemic levels. This study provides evidence that long-term mild diabetes amplifies the alterations found in urethral tissue with severe fibrosis, compared with short-term severe diabetes and have important implications for the monitoring and treatment strategies implemented in patient with *diabetes mellitus*.

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7. COMPETING INTERESTS

The authors declare that there are no competing interests.

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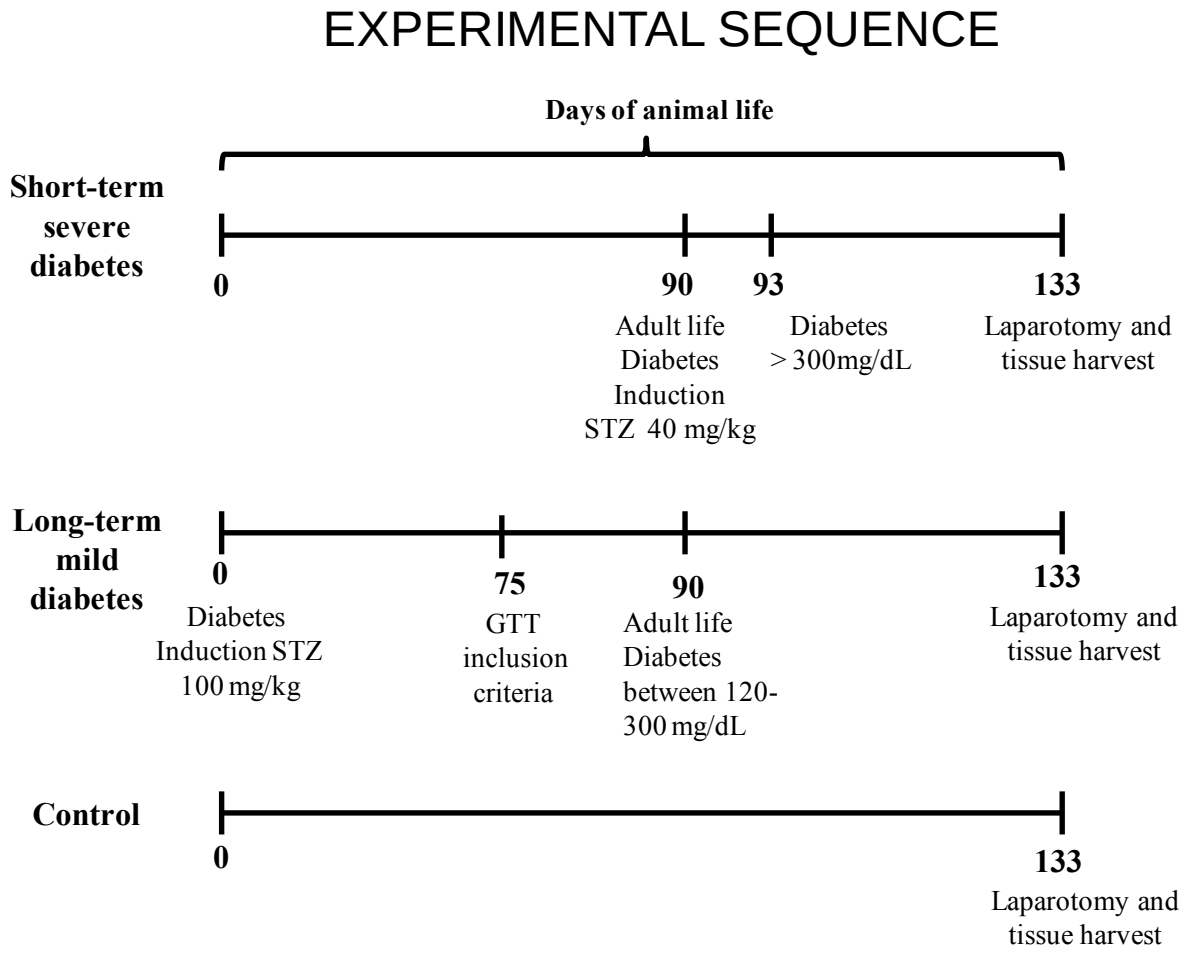

Figure 1. Experimental sequence of all groups.

Table 1. Morphological changes of urethral tissue in all groups.

	CONTROL	LONG-TERM MILD DIABETES	SHORT-TERM SEVERE DIABETES
Pathological analysis (Fibrosis)	- Minimal - Minimal to moderate	- Minimal - Minimal to moderate - Moderate - Severe	- Minimal - Minimal to moderate - Moderate
Morphometric analysis	- Well-organized tissue	↑↑↑ Total collagen area ↓ Striated muscle area	↓ Total collagen area ↓↓↓ Striated muscle area ↑↑ Blood vessels
Immunohistochemical	- Fast myosin heavy chain isoform predominantly - Fast fibers present throughout the outer circular layer - Slow fibers present throughout the inner circular layer	- Loss of specific localization for each fiber type - Greater quantity of slow fibers in the inner layer. - Significantly decreased of fast fibers	- Loss of specific localization for each fiber type - Greater quantity of slow fibers in the inner layer - Significantly decreased of fast fibers
Ultrastructural analysis	- Well-organized myofibrils - Collagen normally distributed	- ↑↑ Interstitial collagen - ↑↑ Lipid droplets - ↑ Mitochondria - ↑ Glycogen granules	- Centrally myonuclei presence

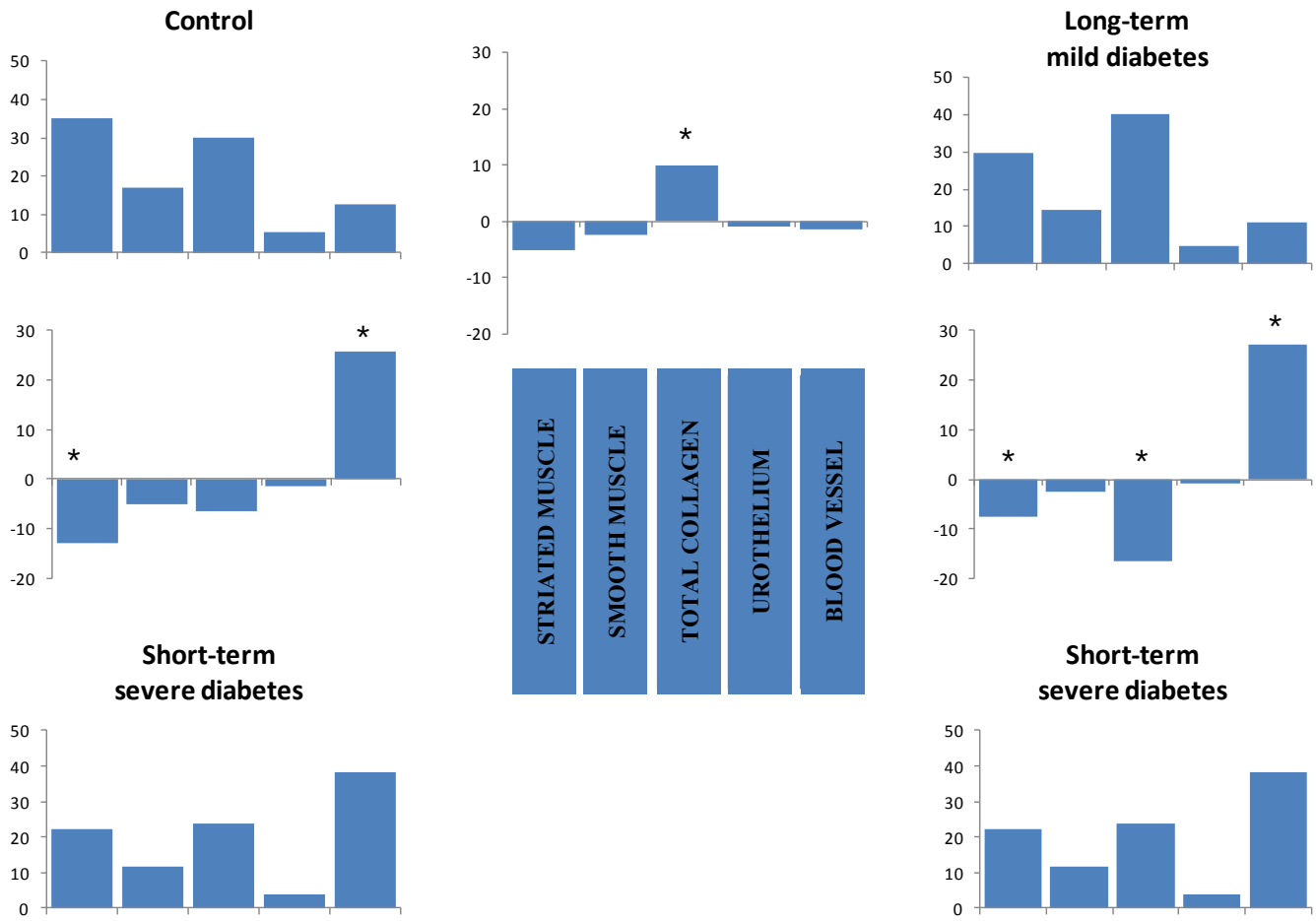

Figure 2. The plot shows how the percentage of each tissue type changes among the groups.

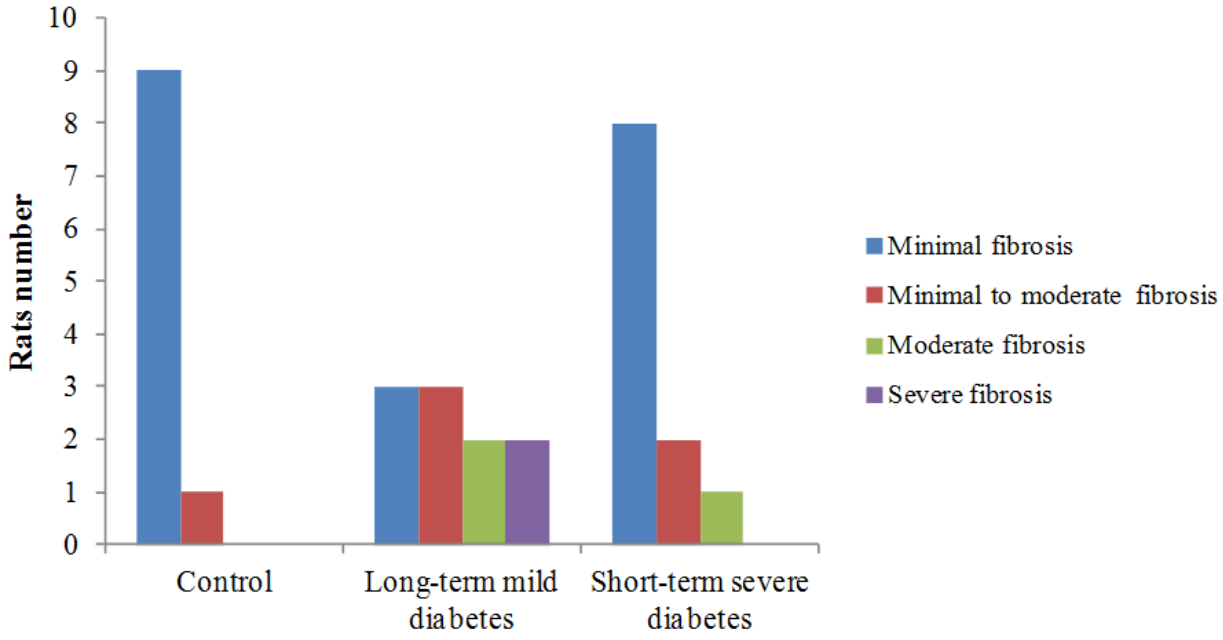

Figure 3. Pathological analysis for the grade of fibrosis in all groups.

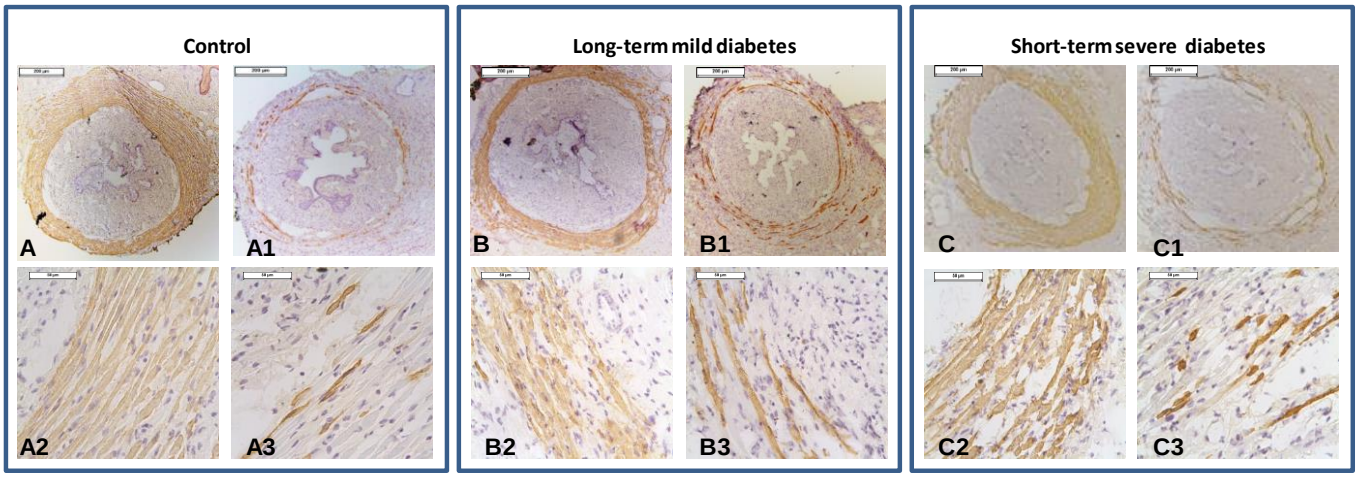

Figure 4. Transverse section of urethra by immunohistochemical staining to fast (A, A2, B, B2, C, C2) and slow (A1, A3, B1, B3, C1, C3) fibers in all groups. Magnification x4 and x40.

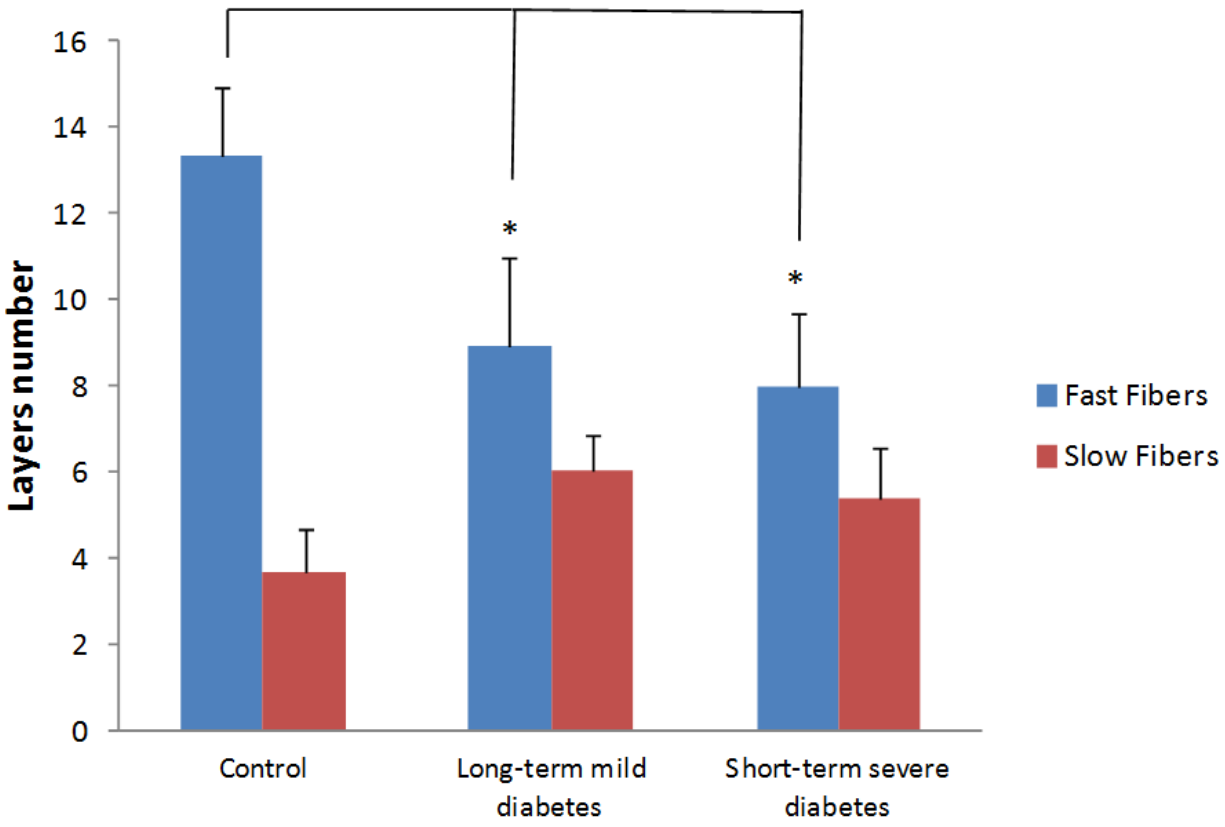

Figure 5. The analysis of fast and slow layers number.

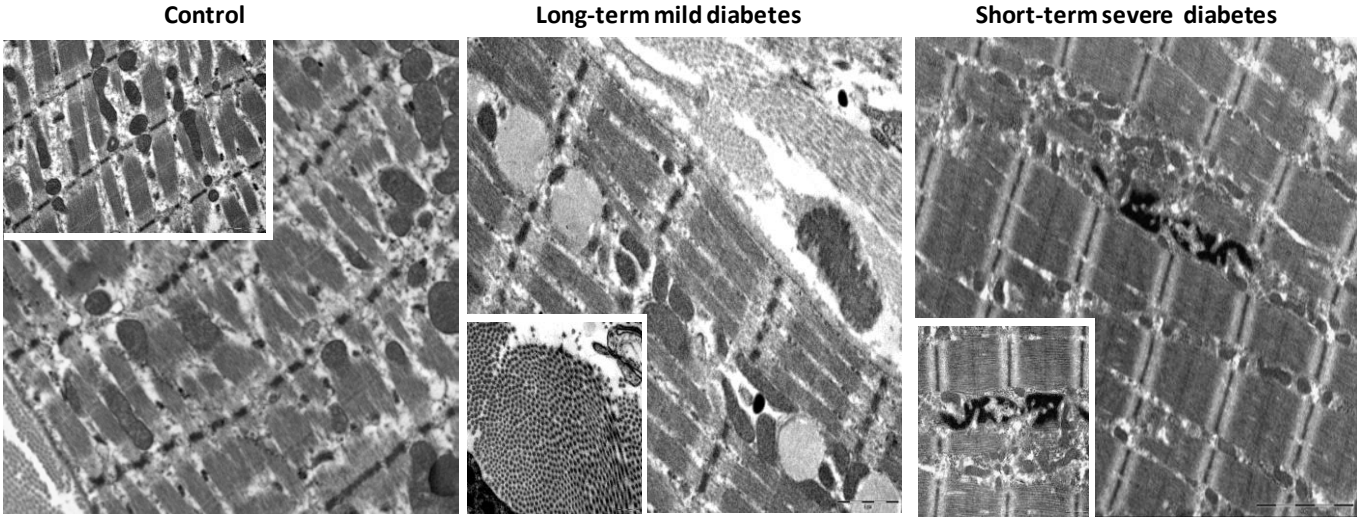

Figure 6. Electron microscopy of urethral striated muscle in all groups.

Capítulo 4

EFEITOS DE DOIS MODELOS DE DIABETE INDUZIDO POR *STREPTOZOTOCIN* NO TECIDO URETRAL DE RATAS PRENHES: GUIA ILUSTRATIVO

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1. INTRODUÇÃO

O mecanismo de continência urinária normal exige uma coordenação complexa entre bexiga, uretra, músculos pélvicos e tecido conjuntivo (1). O mecanismo intrínseco da continência é formado pelo Esfíncter Uretral Externo (EUE) e pelo Esfíncter Uretral Interno (EUI), enquanto o mecanismo externo é formado pelos músculos pélvicos, órgãos e estruturas de suporte ao redor da uretra (2).

A uretra é um tecido dinâmico composto de músculo estriado e liso e matriz extracelular, que possui a função de manter a continência durante o enchimento vesical (pressão uretral excedendo a pressão vesical) e de ajudar a liberação da urina pela bexiga durante a micção (1). A continência ocorre pelo fato da pressão de fechamento uretral exceder a pressão intravesical (3).

A musculatura estriada da uretra e do assoalho pélvico são responsáveis por um terço da pressão uretral. Outro terço é exercido pelo componente vascular e o terço restante é atribuído à musculatura lisa e ao tecido conjuntivo uretral e para-uretral (3). Assim, não há dúvidas que a integridade do tecido uretral é de extrema importância para os mecanismos de continência (4, 5).

A Incontinência Urinária (IU) feminina é definida como toda perda involuntária de urina (6). É uma condição frequente, de alto custo econômico para o governo e que implica em danos físicos, psicológicos, sociais e pior qualidade de vida para as mulheres (7). Sua prevalência pode chegar a 49,5% dependendo na população estudada e do critério empregado como diagnóstico (8).

As associações entre hiperglicemia e IU e entre gestação e IU estão bem estabelecidas (9, 10), entretanto a associação entre hiperglicemia gestacional e IU é escassa na literatura. Em trabalho clínico, nosso grupo de pesquisa verificou que a prevalência de IU gestacional (diabéticas: 50,8% *vs*. normoglicêmicas: 31,6%) e IU dois anos pós-parto cesárea (diabéticas:

44,8% *vs.* normoglicêmicas: 18,4%) foi significativamente mais elevada entre mulheres com *diabetes mellitus* gestacional (DMG) do que entre as normoglicêmicas (11).

A pesquisa translacional em ratas diabéticas prenhes iniciou com trabalho de diabete de intensidade severa (glicemia maior que 300mg/dL), e demonstrou adelgaçamento e atrofia do músculo estriado uretral associado à desorganização e rompimento das fibras. A análise imunoistoquímica evidenciou perda da localização anatômica normal das fibras com diminuição na proporção de fibras rápidas (12).

No segundo trabalho, com o intuito de mimetizar o diabete com intensidade glicêmica moderada (glicemia entre 120-300mg/dL), verificamos que além das alterações musculares, foram encontradas mudanças significativas na matriz extracelular como fibrose e na ultraestrutura como acúmulo de mitocôndrias, aumento de gotas de lipídios e acúmulo de grânulos de glicogênio no grupo diabético prenhe (13).

A heterogeneidade dos sintomas clínicos do sistema urinário, pode resultar de diversos fatores, incluindo a duração e o tipo de diabete (14). A indução do diabete experimental por drogas citotóxicas como o *streptozotocin* (STZ) é bem caracterizado na literatura (15). Dependendo da linhagem do animal utilizado, dose, via de administração da droga e do período de vida em que o STZ é administrado em ratos de laboratório, diferentes intensidades glicêmicas são atingidas: diabete grave (glicemia superior a 300 mg/dL) (16-20) ou o diabete moderado (glicemia entre 120 e 300 mg/dL) (21-24). O diabete grave leva a altos níveis glicêmicos de forma aguda e intensa e reproduz a glicemia de indivíduos com diabete descompensado (DM tipo 1), enquanto o diabete moderado, com ação crônica e intensidade média, reproduz a glicemia vista em mulheres com DMG e/ou clínico DM tipo 2 (13).

Assim, a partir destes estudos translacionais conduzidos por nosso grupo de pesquisa, foi possível verificar diferentes alterações no tecido uretral dependentes do modelo, da intensidade da glicemia e do tempo do diabete (12, 13). Deste modo, diante da escassa literatura sobre as

alterações uretrais em ratas para estudo dos mecanismos que levam a incontinência urinária, e estudos que comparem diferentes modelos de diabete, tornou-se primordial a elaboração de um material-base que abordasse diferentes métodos e resultados para servir de guia para outros pesquisadores. Diferente do atlas da Dissertação de Mestrado de Piculo em 2013 (25) que abordou a estrutura do grupo virgem (controle) com diferentes técnicas, este trabalho detalha as alterações uretrais encontradas frente a prenhez e aos modelos de diabete grave e moderado. Também serve de guia para mostrar as dificuldades e o número de animais e de lâminas que precisam ser usados para obtenção de tamanho amostral que seja adequado.

Deste modo, o objetivo deste trabalho foi construir uma fonte compreensível na avaliação e comparação das estruturas que compõem a uretra em dois modelos experimentais de diabete induzido por *streptozotocin.*

Os tópicos incluem as sequências experimentais do diabete grave e moderado e as diferentes análises histológicas, imunoistoquímicas e ultraestruturais da uretra de ratas prenhes.

2. MATERIAIS E MÉTODO

Ratos (machos e fêmeas) da linhagem *Wistar* foram criados e mantidos no Laboratório de Pesquisa Experimental de Ginecologia e Obstetrícia da Faculdade de Medicina de Botucatu – UNESP. Esses animais foram mantidos (4 animais/caixa) sob condições controladas de temperatura (22 ± 3ºC), umidade (50 ± 10%), ciclo claro/escuro (12h) e com água e ração (Purina®, Brasil) oferecidas *ad libitum.*

Para estudar os modelos de diabete de intensidade de glicemia diferentes, dois estudos paralelos foram conduzidos. Foram formados 8 grupos de 33 animais: grupo virgem controle do diabete moderado, grupo prenhe controle do diabete moderado, grupo diabético moderado, grupo diabético moderado prenhe, grupo virgem controle do diabete grave, grupo prenhe controle do diabete grave, grupo diabético grave e grupo diabético grave prenhe.

Indução do diabete moderado

Após acasalamento de ratas adultas não diabéticas, seus recém-nascidos foram pesados e as fêmeas foram utilizadas no primeiro dia de vida para indução do diabete (grupos diabéticos). A indução foi feita pela administração de *streptozotocin* (STZ – SIGMA Chemical Company, St. Louis, Millstone, EUA), por via subcutânea, na dose de 100 mg/kg de peso corporal diluído em 0,1 mol/l de tampão citrato (pH 4,5) (22). Os RN fêmeas dos grupos não-diabéticos receberam volume do veículo (tampão citrato) correspondente aos RN do grupo STZ. Após a indução, as recém-nascidas foram mantidas com suas mães (máximo de oito fêmeas) até o término do período de amamentação (21 dias). Ao final deste período, as ratas-mães foram mortas com tiopental sódico (Thiopentax® - Cristália Produtos Químicos e Farmacêuticos Ltda., São Paulo, Brasil) na dose de 50 mg/kg de peso corpóreo e as descendentes fêmeas foram mantidas no Biotério do Laboratório de Pesquisa Experimental em Ginecologia, Obstetrícia e Mastologia sob condições controladas.

Indução do diabete grave

Na fase adulta (em torno de 90 dias de idade), ratas normoglicêmicas foram escolhidas aleatoriamente para indução do diabete. Para isto, receberam droga beta citotóxica - *Streptozotocin* (SIGMA - Chemical Company, St. Louis, MO, USA) diluída em tampão citrato (0,1M; pH 4,5) na dose de 40mg/Kg de peso corpóreo, via intravenosa (veia cauda). As ratas dos grupos não-diabéticos receberam pela mesma via de administração tampão citrato (veículo) em volume similar ao recebido pelas ratas dos grupos diabéticos. A seguir, as ratas foram mantidas em gaiolas individuais até a manhã do 7º dia após a indução. A glicemia foi determinada colhendo-se uma gota de sangue por punção com agulha na parte distal da cauda da rata e depositando-a em glicofita. As glicofitas foram lidas em glicosímetro específico (One Touch Ultra – Johnson & Johnson®) para determinação glicêmica e os valores foram expressos em

miligramas por decilitro (mg/dL). O critério de inclusão estabelecido para compor os grupos com diabete grave consistiu em ratas que apresentaram valores glicêmicos superiores a 300 mg/dL (26).

Período de acasalamento e prenhez – Grupos prenhe, diabético moderado prenhe e diabético grave prenhe

Na vida adulta dessas fêmeas (em torno de 90 dias), foi iniciada a fase de acasalamento com duração máxima de 15 dias. No final da tarde, cada quatro ratas fêmeas foram colocadas com um macho em gaiolas de polietileno. Na manhã subsequente, os machos foram retirados e foram coletados os esfregaços vaginais das ratas para análise microscópica. O fator indicativo de prenhez foi a presença de espermatozóides, sendo este considerado dia 0 de prenhez (27, 28).

Durante a prenhez, as fêmeas foram mantidas em gaiolas individuais e nas manhãs dos dias 0 e 21 de prenhez, as glicemias foram mensuradas e os valores foram expressos em miligramas por decilitro (mg/dL).

Coleta do material

Na manhã do dia 21° de experimento, as ratas foram anestesiadas com tiopental sódico e posicionadas em decúbito dorsal em goteira cirúrgica e imobilizadas. Foi realizada depilação da região inguinal, faces interna e posterior das coxas e da região genital; e a seguir incisão em "Y" invertido, iniciando-se no 1/3 caudal da linha mediana do abdome e, na altura da sínfise púbica, bifurcando-se para a face interna das coxas. Para as ratas prenhes, além destes procedimentos, foi realizada laparotomia para exposição dos cornos uterinos para retirada da ninhada.

Posteriormente, a pele dos animais foi rebatida para expor a porção caudal da parede ventro-anterior do abdome, da região inguinal, das faces internas e posteriores das coxas e da genitália externa para remoção do tecido conjuntivo fibroadiposo da região ventral abdominal,

inguinal e perigenital até a face posterior das coxas. Foi feita a dissecação fina da uretra (ventral) e da vagina (dorsal) em conjunto com remoção do tecido conjuntivo e exposição da túnica muscular superficial desses órgãos e remoção do óstio vaginal. A uretra e a vagina foram retiradas em monobloco por meio de secção na porção mais cranial possível junto à sínfise púbica.

Métodos histológicos, imonoistoquímicos e ultraestruturais para avaliação dos componentes uretrais

Microscopia fotônica convencional (n= 10 animais/grupo)

Após a fixação inicial, o conjunto uretra-vagina foi dissecado, retirado, reduzido na região do terço médio e fixado em solução de paraformaldeído. Em seguida, foi lavado em água corrente por 24 horas e foi realizada desidratação em alcoóis de concentrações crescentes, clarificação em xilol e inclusão em paraplast, conforme a técnica de rotina do laboratório de Histologia. A microtomia consistiu de cortes transversais seriados com 4 μm de espessura.

Esses cortes foram submetidos às seguintes colorações: Hematoxilina e Eosina para análise geral das estruturas; Tricrômico de Masson, para análise geral simultânea das fibras musculares e das fibras colágenas; Picrosirius Red 0,1% em solução saturada de ácido pícrico, para análise das fibras de colágeno tipo I e tipo III simultaneamente e para as análises morfométricas; Reticulina de Gomori, para análise das fibras de colágeno tipo I (coradas em dourado) e tipo III (impregnadas pela prata e coradas em preto) separadamente; PAS, para análise dos açúcares neutros presentes nas glicoproteínas das membranas basais das fibras musculares, e Azul de Toluidina 0,025% em HCl 0,1N para evidenciar os glicosaminoglicanos.

Imunoistoquímica dos tipos de fibras musculares (MHC *fast* e MHC *slow*) (n= 10 animais/grupo)

Para a preparação da reação imunoistoquímica, o conjunto uretra-vagina foi exposto, dissecado, retirado e envolvido em talco neutro. A seguir, foi congelado em nitrogênio líquido e armazenado em freezer a -80°C. Posteriormente, foram obtidos cortes histológicos sequenciais em criostato Leica CM 1800 à temperatura de -25° C com espessura de 6 μm.

As lâminas obtidas foram submetidas ao método de imunoperoxidase StreptABComplex/HRP através da utilização de anticorpos comerciais primários específicos para fibras rápidas (1:160) e lentas (1:120) Novocastra.

Microscopia Eletrônica de Transmissão (n=3 animais/grupo)

Após a fixação inicial, o conjunto uretra-vagina foi dissecado, retirado, reduzido às extremidades e foi fixado por 12 horas a 4°C com vermelho de rutênio 0,1%, glutaraldeído 3% em 0,1M de tampão cacodilato (pH 7,4). As amostras então foram lavadas com tampão cacodilato contendo a mesma concentração do corante (3x / 20 min). As amostras coradas com vermelho de rutênio foram fixadas com 1% de tetróxido de ósmio em tampão cacodilato contendo 0,1% de vermelho de rutênio e lavadas com água destilada (3x/ 20 min). Desidratados em sequência crescente de soluções de acetona, embebidos em mistura de resina (Araldite®) e acetona 100% por 12 horas e em resina na estufa a 37˚C por 1 hora. Em seguida, o material foi incluído em resina e a polimerização ocorreu em estufa a 60˚C por 72 horas.

Após inclusão do material em araldite, foi realizada a trimagem e a seleção de campos desejados, através de cortes semi-finos corados em mistura de azul de metileno 1% e azul II 1% em solução de bórax 1%. Os cortes ultra-finos foram feitos em ultramicrótomo Ultratome III 880 da LKB. Para analisar a qualidade da técnica, o material foi conduzido e visualizado no

microscópio eletrônico de transmissão (Philips CM 100) do Centro de Microscopia Eletrônica do Instituto de Biociências da UNESP de Botucatu.

Análise do material

Os cortes das uretras foram analisados qualitativamente, de acordo com o objetivo de cada método histológico utilizado.

3.RESULTADOS

O número total de animais que iniciaram a pesquisa nos dois experimentos foram de 549 ratas. Entre estas, 322 do experimento de diabete grave e 227 do experimento de diabete moderado. Destas, 70 animais fizeram parte dos grupos virgens não diabéticos, 136 dos grupos prenhes não diabéticos, 219 dos grupos com diabete grave e 124 dos grupos com diabete moderado. No final dos experimentos, os grupos foram formados com 33 animais por grupo, o que demonstra grande dificuldade na obtenção dos modelos de diabete e prenhez.

Após a obtenção do material de estudo, foram confeccionadas 2400 lâminas para os materiais incluídos em parafina e 1200 lâminas para os materiais congelados em nitrogênio líquido, perfazendo um total de 3600 lâminas.

Os grupos não diabéticos virgens e prenhes apresentaram resultados similares e foram analisados de forma conjunta.

Resultados da Microscopia Fotônica Convencional:

Através de cortes transversais corados com **Hematoxilina-eosina** (HE), foi possível observar a morfologia geral da uretra, que mostrou-se normal em todos os grupos estudados, onde foi observada: luz uretral irregular parcialmente colabada, constituída de várias pregas de mucosa formada por epitélio estratificado pavimentoso e lâmina própria/submucosa de tecido

conjuntivo frouxo. Duas camadas de músculo liso envolviam as túnicas mucosa e submucosa sendo uma com orientação longitudinal (interna) e outra circular (externa). Em vários pontos um entrelaçamento destas camadas foi observado. Mais externamente uma camada de fibras musculares estriadas orientada circularmente circunscrevia a uretra ao longo de toda sua extensão. Essa camada corresponde ao músculo uretral externo. Entre as camadas de músculo liso e estriado estava presente um plexo vascular (Figuras 1-7).

A coloração de **Tricômico de Masson**, que permite análise simultânea das fibras musculares (vermelho) e das fibras de colágeno (azul) evidenciou aumento de fibras colágenas nos grupos diabético moderado e diabético moderado prenhe. O músculo uretral constituído por fibras musculares estriadas, mostrou-se mais delgado e desorganizado nos grupos diabéticos. Foi observado grande número de vasos sanguíneos no grupo diabético grave (Figuras 1-7).

Através da coloração de **Picrosírirus Red** e **Reticulina de Gomori** obteve-se a confirmação da maior quantidade de colágeno presente entre as fibras musculares no grupos diabete moderado e diabete moderado prenhe (Figuras 1-7).

A análise dos açúcares neutros presentes nas glicoprotéinas das membranas basais das fibras musculares pode ser observado através da coloração de **PAS**, onde foi detectado aumento na concentração de glicogênio nos grupos prenhes (Figuras 1-7).

Na tentativa de verificar a presença de glicosaminoglicanos sulfatados e carboxilados, foi realizada a coloração de **Azul de Toluidina**, a qual evidenciou discreta quantidade desses polissacarídeos na membrana basal das fibras musculares em todos os grupos estudados (Figuras 1-7).

Resultados da Imunoistoquímica - anticorpos para miosina rápida e lenta

As reações de imunoistoquímica revelaram um predomínio de fibras rápidas no músculo estriado uretral do grupo Virgem. Sendo que essa camada apresentou-se espessa, circundando a região uretral mais externa, enquanto a camada de fibras lentas é delgada e localiza-se mais

internamente (Figura 8). No grupo Prenhe, a distribuição das fibras rápidas e lentas e a proporção entre elas foi similar ao encontrado no grupo Virgem (Figura 9).

Os grupos Diabético Moderado e Diabético Moderado Prenhe não apresentaram a característica de distribuição descrita acima, onde foi identificada uma co-localização das fibras rápidas e lentas, sendo que as fibras rápidas estavam em menor proporção ou quantidade (Figuras 10 e 11). Características imunoistoquímicas similares foram observadas nos Grupos Diabético Grave e Diabético Grave Prenhe (Figuras 12 e 13).

Todos os grupos estão representados na Figura 14.

Resultados da análise ultraestrutural

A análise ultraestrutural, mostrou miofibrilas bem organizadas formando sarcômeros íntegros com características morfológicas relacionadas aos diferentes tipos musculares, sem sinais de alteração no **Grupo Virgem** (Figura 15 A,B,C).

No **Grupo Prenhe**, foi observada a presença de muitos vacúolos contendo figuras de mielina, características de células em degeneração. Grânulos de glicogênio dispersos, apresentaram-se em forma e quantidade usuais (Figura 15 D,E,F).

No **Grupo Diabético Moderado** foi observada maior quantidade de colágeno. Gotículas de lipídeos estavam presentes, especialmente na banda I e entre os miofibrilas onde se acumulam as mitocôndrias (Figura 16 A). Além disso, numerosas mitocôndrias subsarcolemais e intermiofibrilares estavam presentes. Grânulos de glicogênio estavam dispersos aparentemente em maior quantidade (Figura 16 B,C).

O **Grupo Diabético Moderado Prenhe** apresentou características ultraestruturais similares ao grupo diabético moderado (Figura 16 D, E, F).

No **Grupo Diabético Grave** destaca-se a presença de núcleo central (Figura 17 C) e túbulos T.

No **Grupo Diabético Grave Prenhe**, nota-se acúmulo de mitocôndrias subsarcolemais. A observação das fibras colágenas sugere um aumento na sua quantidade entre as fibras musculares (Figura 17 D,E,F).

Todos os grupos estão representados na Figura 18.

4.CONCLUSÃO

Espera-se que este material sirva como instrumento didático prático para guiar outros pesquisadores nos estudos das repercussões dos diferentes modelos de diabete e prenhez na uretra de ratas, e assim seja cada vez maior o entendimento dos mecanismos que levam à incontinência urinária.

ILLUSTRATIVE GUIDE OF URETHRAL TISSUE IN PREGNANT RATS IN TWO MODELS OF DIABETES

1. INTRODUCTION

The normal urinary continence mechanism requires a complex coordination between the bladder, urethra, pelvic muscles and connective tissue (1). The intrinsic mechanism of continence is formed by the external urethral sphincter and the internal urethral sphincter, while the external mechanism is formed by the pelvic muscles, organs and structures around the urethra (2).

The urethra is a dynamic tissue composed striated and smooth muscle and extracellular matrix , which has the function of maintaining continence during bladder filling (urethral pressure exceeds the bladder pressure), and help the release of the urine bladder during micturition (1). Continence occurs because the urethral closure pressure exceeds intravesical pressure (3).

The urethral striated muscles and pelvic floor are responsible for one third of the urethral pressure. Another third is held by the vascular component and the remaining third is attributed to the urethral smooth muscle and connective tissue (3). Thus , there is no doubt that the urethral integrity is importance for continence mechanisms (4, 5).

Urinary incontinence (UI) is defined as any female involuntary loss of urine (6). It is a common condition, with high cost to the government and that implies physical, psychological , social damage and poorer quality of life for women (7). Its prevalence can reach 49.5 % depending on the population studied and the criteria used for diagnosis (8).

The associations between hyperglycemia and UI and UI and pregnancy are well established (9, 10) however the association between gestational hyperglycemia and UI is scarce

in the literature. In clinical work, our research group found that the prevalence of gestational UI (50.8% diabetic vs 31.6 % normoglycemic) and UI two years post- cesarean delivery (44.8 % diabetic vs 18.4% normoglycemic) was significantly higher among women with gestational diabetes mellitus than among normoglycemic (11).

Translational Research in diabetic pregnant rats began with severe diabetes (blood glucose greater than 300mg/dL), and demonstrated thinning and atrophy associated with disorganization and disruption of urethral striated muscle fibers. Immunohistochemical analysis showed loss of normal anatomic location of the fibers with a decrease in the proportion of fast fibers (12).

In the second study, in order to mimic diabetes with moderate intensity (blood glucose between 120-300mg/dL), we found significant changes in the extracellular matrix such as fibrosis and accumulation of mitochondrial, increased of lipid droplets and accumulation of glycogen granules in the diabetic pregnant group (13).

The heterogeneity of clinical symptoms of the urinary system, can result from several factors , including the duration and type of diabetes (14). Induction of experimental diabetes by cytotoxic drugs such as *streptozotocin* (STZ) is well characterized in the literature (15). Depending on the strain of animal used , dose, route of drug administration and the period of life in which STZ is administered in rats, glucose different intensities are achived: severe diabetes (blood glucose above 300 mg/dL) (16-20) or mild diabetes (blood glucose between 120 and 300 mg/dL) (21-24). Severe diabetes leads to high glucose levels in acute and intense and reproduces blood glucose of patients with uncontrolled diabetes (type 1 DM), while mild diabetes, chronic mild intensity action and reproduces blood glucose seen in women with GDM and/or clinical DM2 (13).

Thus, from studies conducted by our research group, we observed different changes in urethral tissue dependent on the model, the intensity and time of diabetes (12, 13). Therefore, the

scarce literature on urethral changes in rats to study the mechanisms that lead to urinary incontinence, and studies comparing different models of diabetes, has become important preparing a base material that addressed different methods and results for serve as a guide for other researchers. Different from Master's Dissertation of Piculo in 2013 (25) that addressed the structure of the virgin group (control) with different techniques, this work will detail other urethral abnormalities found in pregnancy and in models of severe and mild diabetes.

The objective of this material was to build an understandable source for evaluation and comparison of the urethral structures in two experimental models of diabetes induced by *streptozotocin.*

Topics include the experimental sequences of severe and mild diabetes and the different histological, immunohistochemical and ultrastructural analysis of the urethra in pregnant rats.

2. MATERIALS AND METHODS

Female and male Wistar rats, 12-13 weeks of age, were housed in a certified animal care facility and food and water were provided *ad libitum*. All experimental procedures were approved by the Ethics Committee on Animal Experiments of the Botucatu Medical School – UNESP (Protocol Number 846). Rats were maintained under controlled conditions (temperature 22 ± 2°C, humidity 55 ± 5% and 12h light/dark cycle). Eight groups with 33 animals/group were composed: virgin group control of severe diabetic, pregnant group control of severe diabetic, severe diabetic group, severe diabetic pregnant group, virgin group control of mild diabetic, pregnant group control of mild diabetic, mild diabetic group and mild diabetic pregnant group.

Pictures from virgin group was part of the Piculo's Master Dissertation in 2013 (25).

Mild Diabetes

Parental non-diabetic female rats were mated with non-diabetic males to obtain female newborns. For the induction of mild diabetes (blood glucose between 120-300 mg/dL), female newborns of non-diabetic female rats received *streptozotocin* (STZ - 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 (29). 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. Newborn rats remained with their mothers until day 21 of life (weaning period). Glucose tolerance test (GTT) was performed at day 75 of life according to Campos et al. (30) to assess the development of altered glucose metabolism and used as a criterion for include the rats in the certain group. For rats that presented glycemia higher than 140 mg/dL in more than two measures during GTT continued in the experiment. Glucose responses during the GTT were evaluated by estimation of the total area under the curve, using the trapezoidal method. Other female newborns received only citrate buffer and when glycemic levels were below 120 mg/dL these animals were considered as non-diabetic and included in this study. After these procedures, two initial groups were obtained: diabetic and non-diabetic.

Severe diabetes

Severe diabetes was induced (90 days of age) in rats by *streptozotocin* injection (STZ; SIGMA Chemical Company, St. Louis, MO, USA). The STZ was administered intravenously at 40 mg/kg to produce a permanent and severe diabetic state. Blood samples were taken 72 h after STZ injection to confirm diabetes (blood glucose level >300 mg/dL) (26).

Pregnant period and Tissue Harvest

In pregnant groups, diabetes was induced seven days prior to mating. The female rats were mated overnight with non-diabetic male rats. The morning when sperm were found in the vaginal smear was designated as gestational day 0.

The rats were killed on day 21 of the experimental by i.p. Thiopentax® injection at 80 mg/kg. The offspring were removed, weighed and lethally anesthetized with sodium thiopental (3% Thiopentax®) and the maternal urethrovaginal tissues were harvested (cross-section of the mid-urethra and anterior vagina). The investigators controlled the longitudinal axis (proximal to distal) of the urethra by marking with a permanent ink pen to identify the distal urethra. All the analyses were performed in the same points along the urethral longitudinal axis: mid-urethra region, where the striated muscle layer becomes denser.

Histological examination, immunohistochemical stain, and morphometric analysis

A portion of the samples (N=10 samples/group) was immersed in neutral buffered formalin containing 4% formaldehyde for a period of 4h and embedded in paraffin. Sections of 4 μm thickness were cut in the mid-urethra using a rotor microtome and stained with Hematoxilin & Eosin, Masson's trichrome, Picrosirius red, Gomori's reticulin, PAS and Toluidin blue for morphological analyses.

Additional samples (10 samples/group) were frozen in liquid nitrogen and kept at -80 °C for mid-urethra sectioning in a cryostat (6 μm thick) for immunohistochemical analysis of fast and slow fibers. Antibodies WB-MHCf Novocastra (1:120) and WB-MHCs Novocastra (1:180) were used.

Other samples (3 samples/group) were immersed in the fixative solution containing 0.05% ruthenium red for 3h before post fixation in osmium tetraoxide. After the staining, the usual

procedures for transmission electron microscopy were performed for ultrastructural analysis of urethral striated muscle.

Analysis of the material

Urethras were analyzed qualitatively according to the purpose of each histological method.

3. RESULTS

The total number of animals that initiated the research in two experimental groups were 549 rats. Among these, 322 of severe diabetes study and 227 of the mild diabetes study. 70 animals were part of the non-diabetic virgins groups, 136 were part of the non-diabetic pregnant groups, 219 of the severe diabetic groups and 124 of the mild diabetic groups. At the end of the experiments, groups were formed with 33 animals per group, which showed great difficulty in obtaining models of diabetes and pregnancy.

After obtaining the study material, 2400 slides from materials embedded in paraffin and 1200 slides from frozen materials were made, totaling 3600 slides.

Both non-diabetic virgin and pregnant groups showed similar results and was analyzed together.

Light microscopy results

Through cross-sections stained with **hematoxylin-eosin (HE**), we could observe the general morphology of the urethra , which was normal in all groups studied, where it was observed: irregular urethral lumen partially closed, consisting of several folds of mucous formed by stratified squamous epithelium and lamina propria/ submucosa of loose connective tissue. Two layers of smooth muscle involved the mucosa and submucosa tunics one with (inner) longitudinal orientation and another circular (external). More externally a layer of striated

muscle fibers oriented circumferentially circumscribed the urethra along its entire length. This layer is called the urethral striated muscle. Between layers of smooth and striated muscle was present vascular plexus (Figures 1-7) .

The **Masson's trichrome** staining, which allows simultaneous analysis of muscle fibers (red) and collagen blue) fibers showed an increase in mild diabetic and mild diabetic pregnant groups. The urethral muscle consists of striated muscle fibers, was more thinner and disorganized in diabetic groups. Large number of blood vessels was observed in the severe diabetic group (Figures 1-7).

By staining **Picrosírirus Red and Gomori's Reticulin** obtained the confirmation of the greater amount of collagen present between muscle fibers in mild diabetic and mild diabetic pregnant groups (Figures 1-7).

The analysis of neutral sugars in glycoproteins of basement membranes of muscle fibers can be observed by **PAS staining**, which was detected increase in the concentration of glycogen in pregnant groups (Figures 1-7).

In an attempt to verify the presence of carboxylated and sulfated glycosaminoglycans, **staining of Toluidine Blue** was performed, which showed a mild amount of these polysaccharides in the basement membrane of muscle fibers in all groups studied (Figures 1-7) .

Immunohistochemical results

Virgin Group- The immunohistochemical staining in the virgin group 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. A thin, inner circular layer of slow striated muscle fibers was observed with individual fibers being small and thin. The image shows different localization patterns for each type of fibers, with fast fibers being outermost and slow fibers innermost (Figure 8).

Pregnant Group - The distribution of fast and slow fibers and the proportion between them were similar to those of the virgin group (Figure 9).

Mild Diabetic Group showed that the specific localization for each type of fiber was lost, with co-localization of fast and slow fibers and a decreased proportion from fast to slow fibers (Figure 10, 11).

Mild Diabetic Pregnant Group - The immunohistochemical staining in the diabetic pregnant group 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 (Figure 10, 11).

Severe Diabetic and **Severe Diabetic Pregnant Groups** presented the same founds in Mild Diabetic and Mild Diabetic Pregnant groups (Figure 12, 13).

All groups can be seen in Figure 14.

Ultrastructural analysis results

By qualitative ultrastructural analysis, the **Virgin Group** urethral striated muscle demonstrated well-organized myofibrils forming intact sarcomeres with morphological characteristics that were related to different muscle types (Figure 15 A,B,C).

In the **Pregnant Group**, was observed an increase in mitochondria profile and it is highlighted the presence of myelin figure between the myofibers (Figure 15 D,E,F).

Mild Diabetic Group, increased collagen and lipid droplets were noted, especially in the band I and between myofibrils where mitochondria accumulate. Furthermore, numerous subsarcolemmal and intermyofibrillar mitochondria were apparent in the striated muscle cells. The glycogen granules were dispersed in larger quantities (Figure 16 A,B,C).

Mild Diabetic Pregnant Group, presented similar findings to Mild Diabetic Group (Figure 16 D,E,F).

Severe Diabetic Group showed centrally located myonuclei presence and sarcoplasmic reticulum sparse T tubes (Figure 17 A,B,C).

In the **Severe Diabetic Pregnant Group**, subsarcolemmal mitochondria accumulation were apparent. Collagen fibers were present between muscle fibers, however the analysis in diabetic groups suggests an increase in this quantity. (Figure 17 D,E,F).

All groups can be seen in Figure 18.

4. CONCLUSIONS

It is expected that this guide became a practical material for other researchers to study the impact of different models of diabetes and pregnancy in the urethral tissue of female rats to be increasingly understanding the mechanisms that lead to urinary incontinence.

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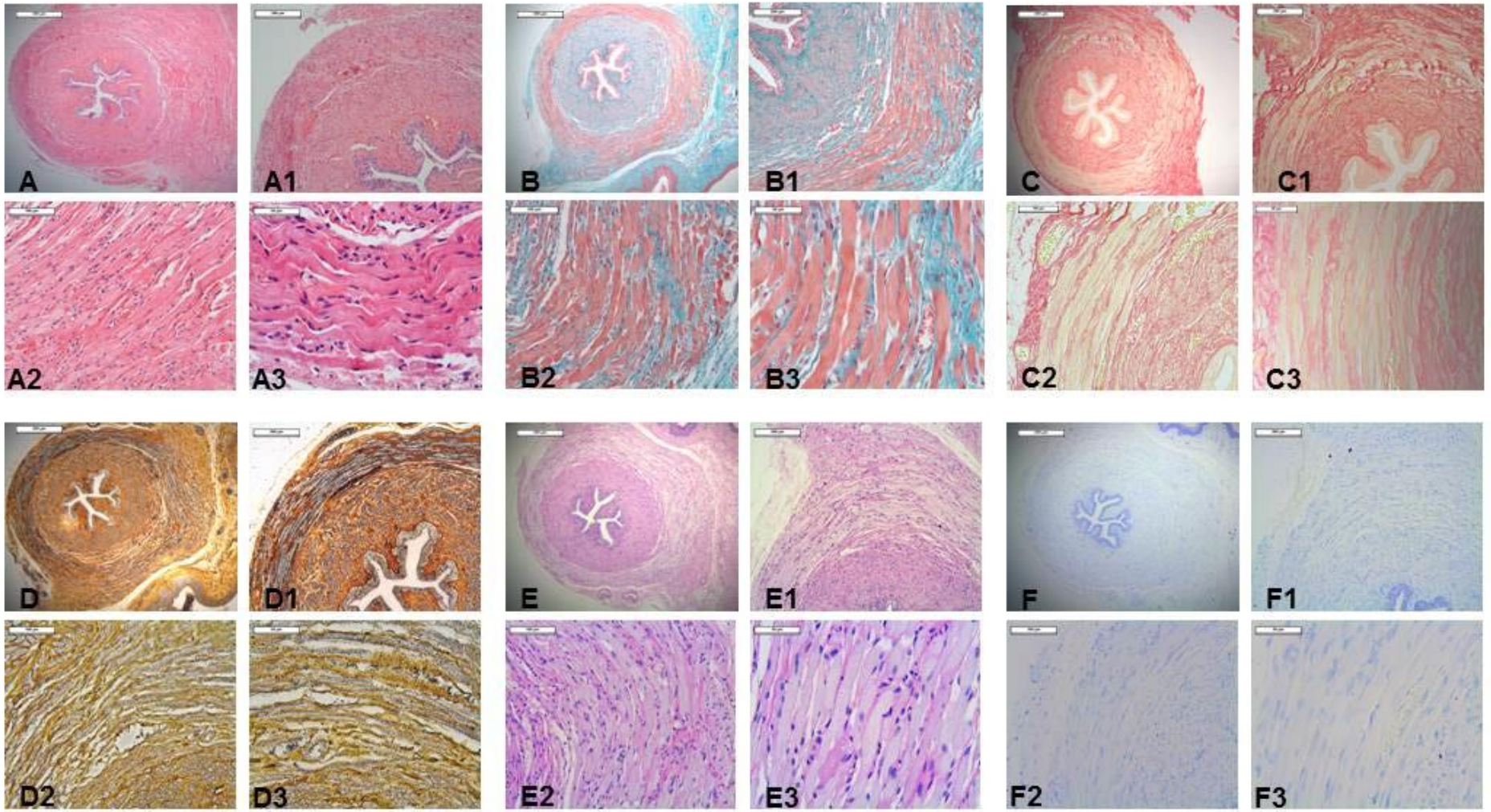

Figura 1. Imagens de cortes transversais das uretras de ratas do grupo Virgem coradas em H&E (A), Tricômico de Masson (B), Picrosírius red (C), Reticulina de Gomori (D), PAS (E) e Azul de Toluidina (F), nos aumentos de 4x (A, B,C,D,E,F) 10x (A1, B1, C1, D1, E1, F1), 20x (A2,B2,C2,D2,E2,F2) e 40x (A3,B3,C3,D3,E3,F3).

Figure 1. Transverse section of urethra in Virgin group by H&E (A), Masson Tricome (B), Picrosirius red (C), Gomori's reticulin (D), PAS (E), Toluidine blue (F) staining. x4, x10, x20 and x40magnfication.

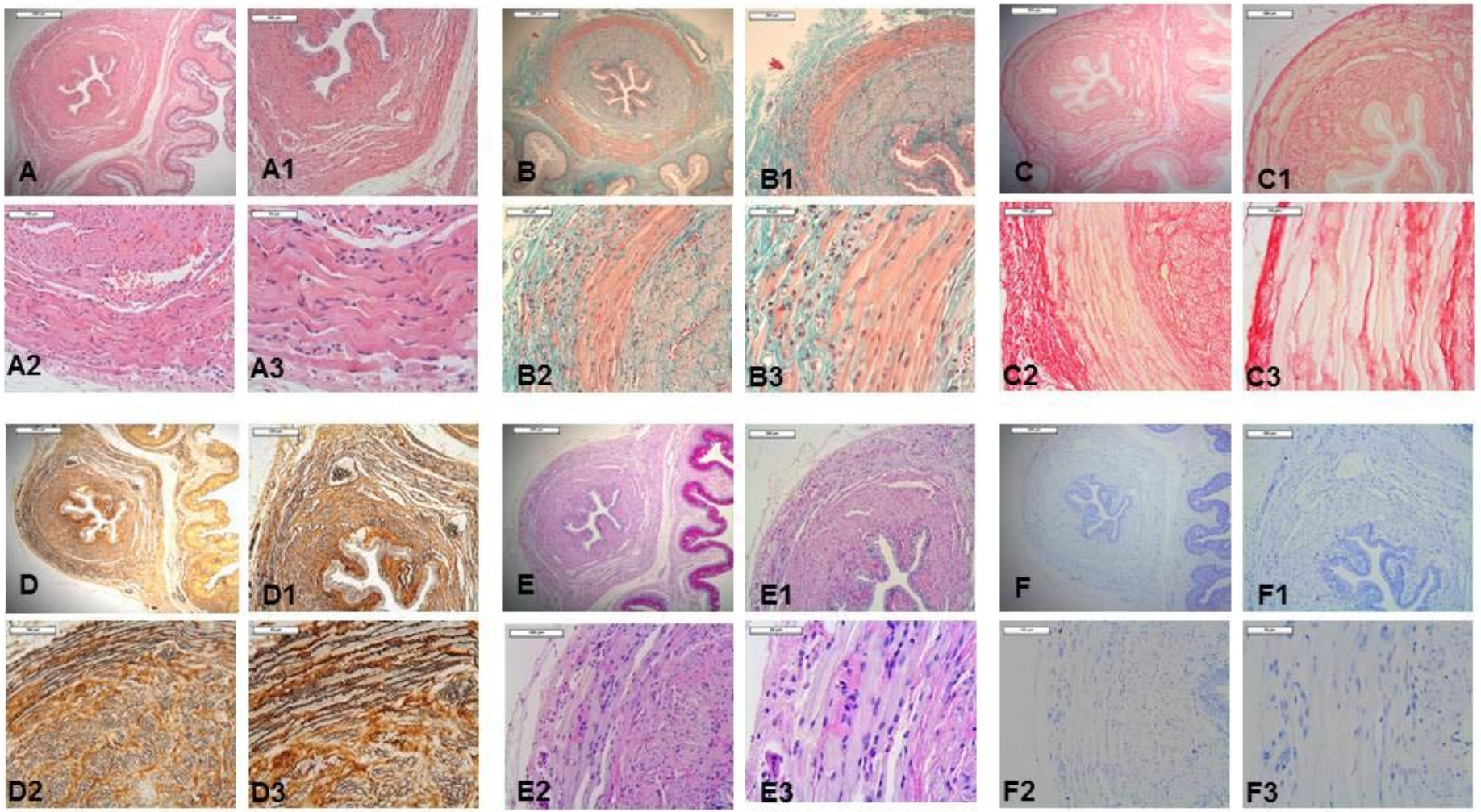

Figura 2. Imagens de cortes transversais das uretras de ratas do grupo Prenhe coradas em H&E (A), Tricômico de Masson (B), Picrosírius red (C), Reticulina de Gomori (D), PAS (E) e Azul de Toluidina (F), nos aumentos de 4x(A, B,C,D,E,F) 10x (A1, B1, C1, D1, E1, F1), 20x (A2,B2,C2,D2,E2,F2) e 40x (A3,B3,C3,D3,E3,F3).

Figure 2. Transverse section of urethra in Prenhe group by H&E (A), Masson Tricome (B), Picrosirius red (C), Gomori's reticulin (D), PAS (E), Toluidine blue (F) staining.

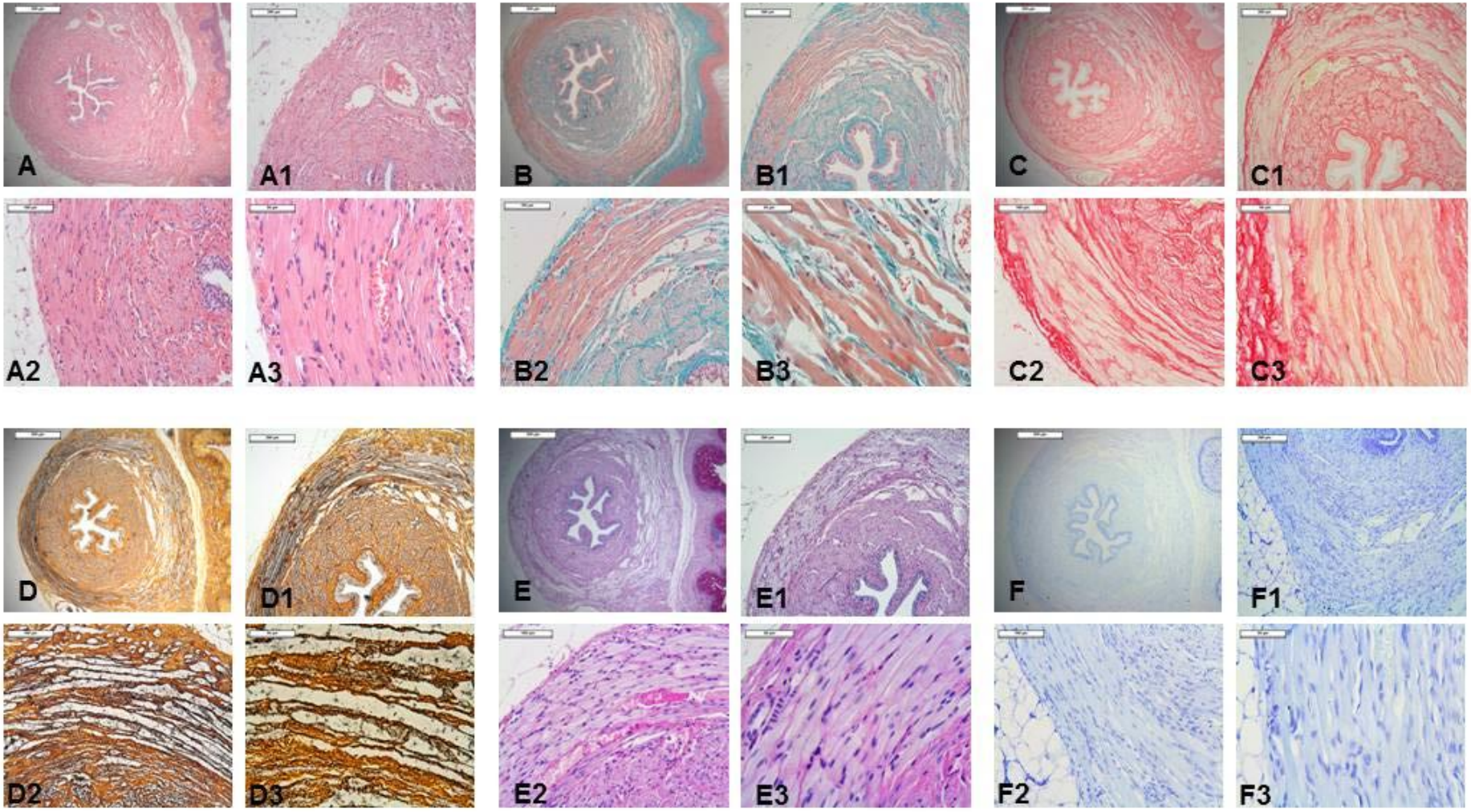

Figura 3. Imagens de cortes transversais das uretras de ratas do grupo Diabético moderado coradas em H&E (A), Tricômico de Masson (B), Picrosírius red (C), Reticulina de Gomori (D), PAS (E) e Azul de Toluidina (F), nos aumentos de 4x(A, B,C,D,E,F) 10x (A1, B1, C1, D1, E1, F1), 20x (A2,B2,C2,D2,E2,F2) e 40x (A3,B3,C3,D3,E3,F3).

Figure 3. Transverse section of urethra in Mild Diabetic group by H&E (A), Masson Tricome (B), Picrosirius red (C), Gomori's reticulin (D), PAS (E), Toluidine blue (F) staining.

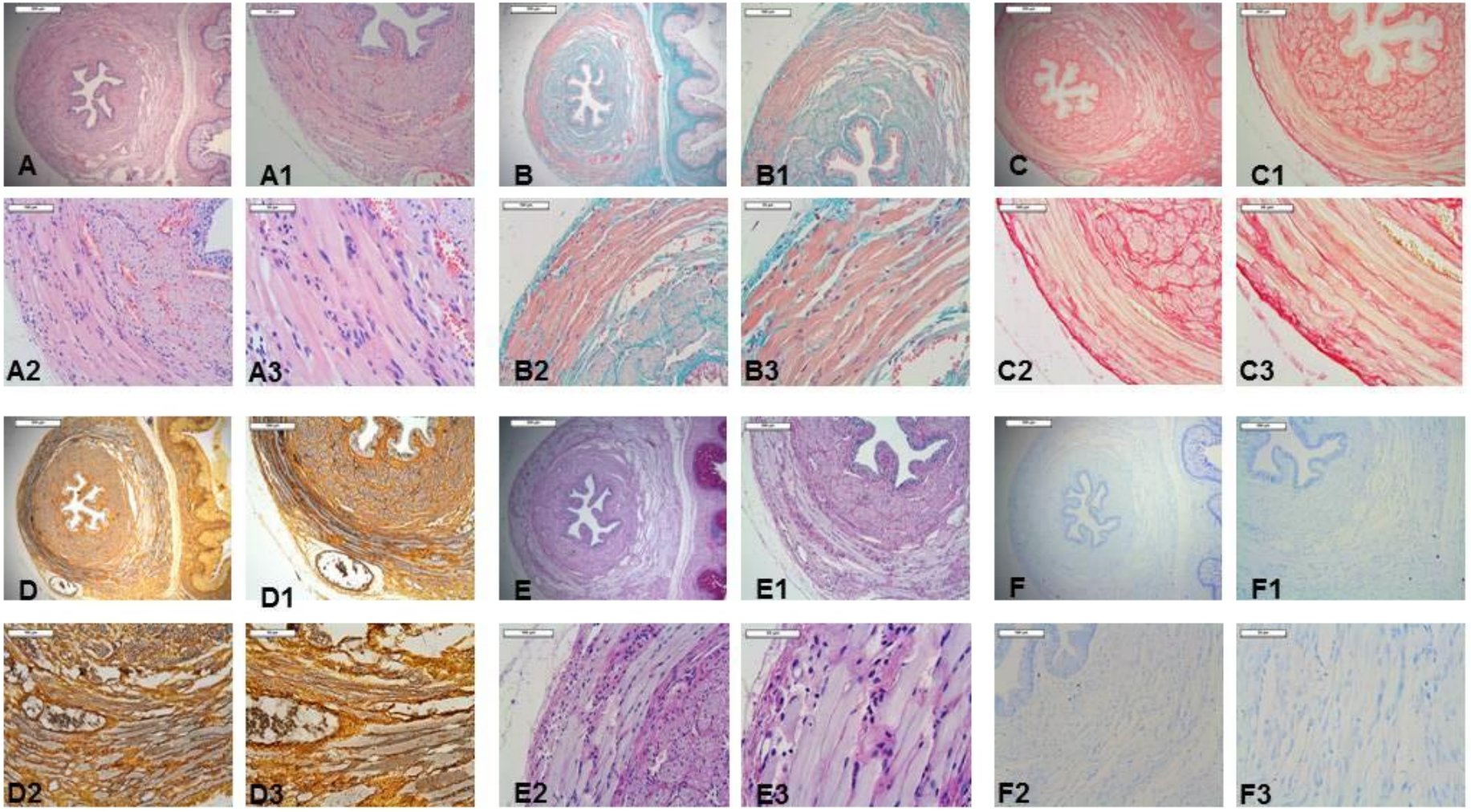

Figura 4. Imagens de cortes transversais das uretras de ratas do grupo Diabético Moderado Prenhe coradas em H&E (A), Tricômico de Masson (B), Picrosírius red (C), Reticulina de Gomori (D), PAS (E) e Azul de Toluidina (F), nos aumentos de 4x(A, B,C,D,E,F) 10x (A1, B1, C1, D1, E1, F1), 20x (A2,B2,C2,D2,E2,F2) e 40x (A3,B3,C3,D3,E3,F3).

Figure 4. Transverse section of urethra in Mild Diabetic Pregnant group by H&E (A), Masson Tricome (B), Picrosirius red (C), Gomori's reticulin (D), PAS (E), Toluidine blue (F) staining.

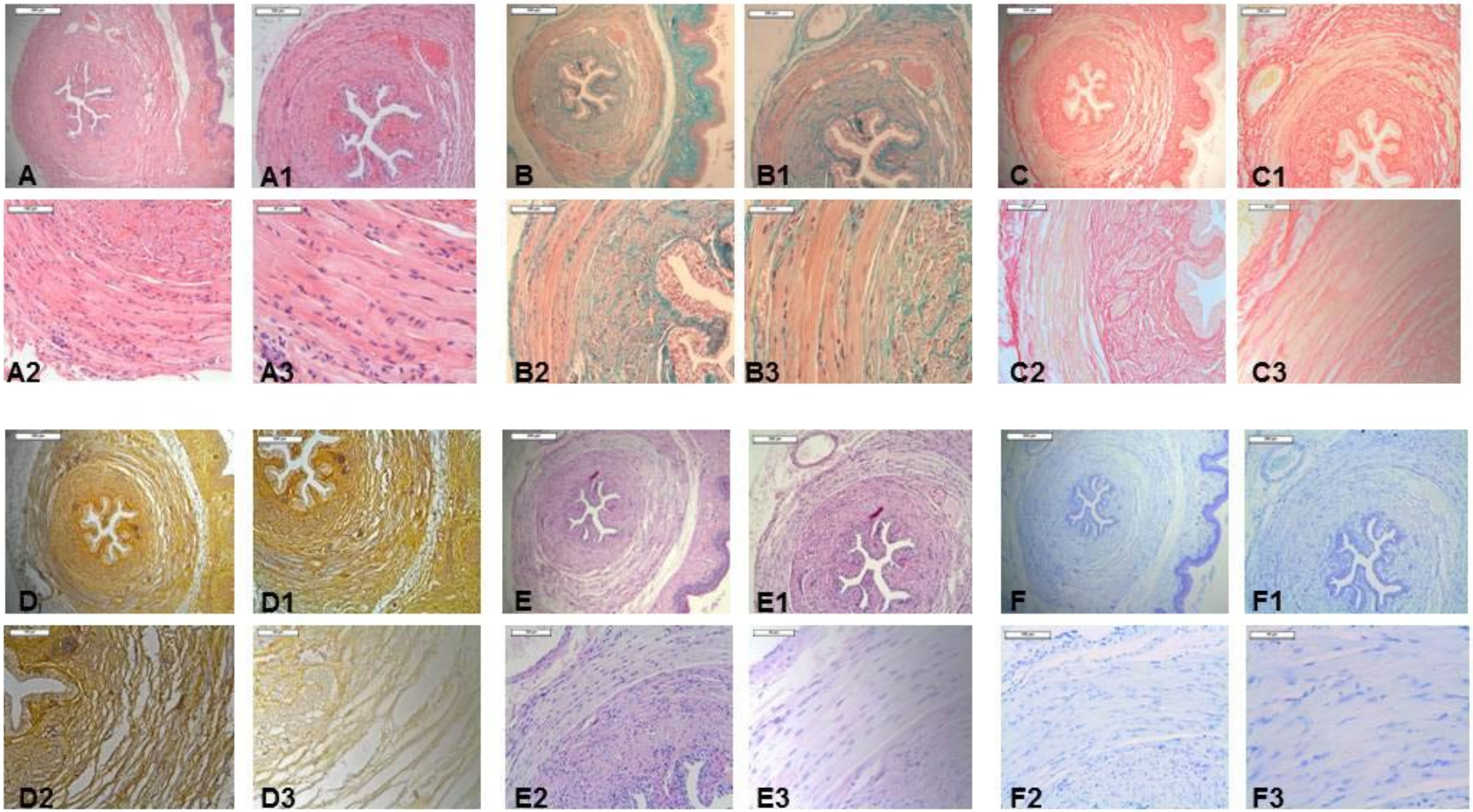

Figura 5. Imagens de cortes transversais das uretras de ratas do grupo Diabético Grave coradas em H&E (A), Tricômico de Masson (B), Picrosírius red (C), Reticulina de Gomori (D), PAS (E) e Azul de Toluidina (F), nos aumentos de 4x(A, B,C,D,E,F) 10x (A1, B1, C1, D1, E1, F1), 20x (A2,B2,C2,D2,E2,F2) e 40x (A3,B3,C3,D3,E3,F3).

Figure 5. Transverse section of urethra in Severe Diabetic group by H&E (A), Masson Tricome (B), Picrosirius red (C), Gomori's reticulin (D), PAS (E), Toluidine blue (F) staining.

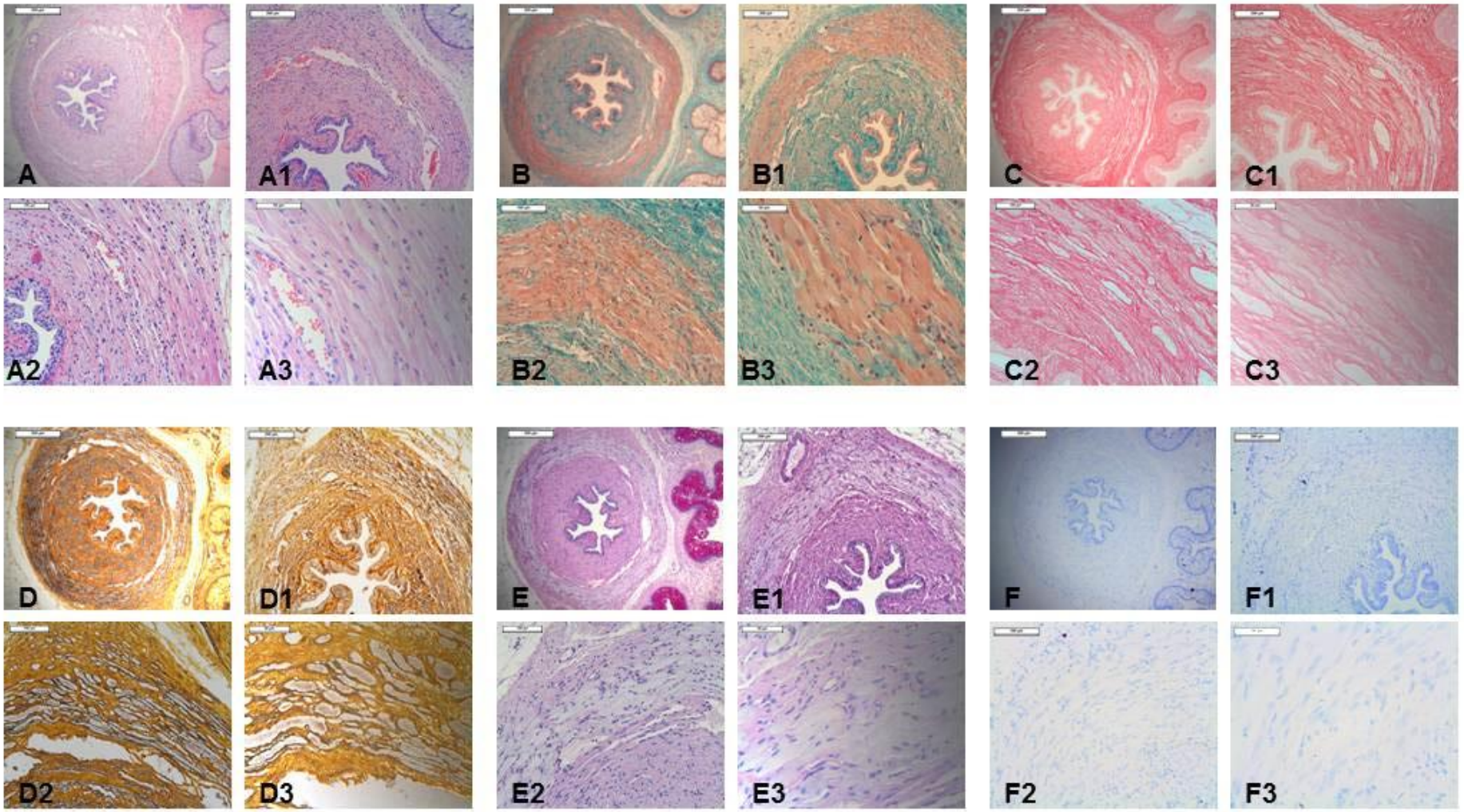

Figura 6. Imagens de cortes transversais das uretras de ratas do grupo Diabético Grave Prenhe coradas em H&E (A), Tricômico de Masson (B), Picrosírius red (C), Reticulina de Gomori (D), PAS (E) e Azul de Toluidina (F), nos aumentos de 4x(A, B,C,D,E,F) 10x (A1, B1, C1, D1, E1, F1), 20x (A2,B2,C2,D2,E2,F2) e 40x (A3,B3,C3,D3,E3,F3).

Figure 6. Transverse section of urethra in Severe Diabetic Pregnant group by H&E (A), Masson Tricome (B), Picrosirius red (C), Gomori's reticulin (D), PAS (E), Toluidine blue (F) staining.

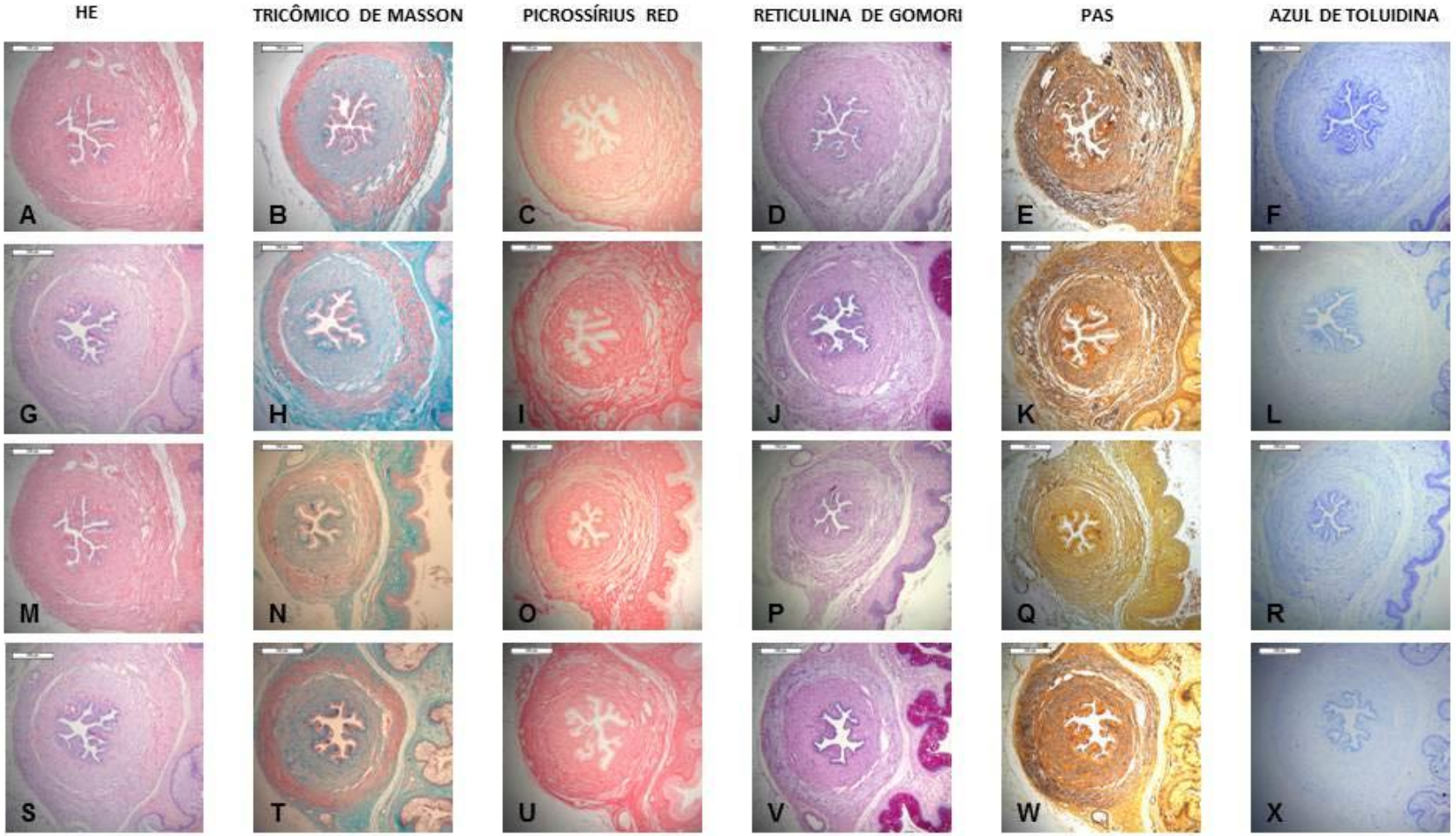

Figura 7. Imagens de cortes transversais das uretras de ratas do grupo Diabético Moderado (A, B, C,D,E,F), Diabético Moderado Prenhe (G, H, I, J, K,L), Diabético Grave (M,N,O,P,Q,R) e Diabético Grave Prenhe (S,T,U,V,W,X) .

Figure 7. Transverse section of urethra in Mild Diabetes (A, B, C,D,E,F), Mild Diabetic Pregnant (G, H, I, J, K,L), Severe Diabetic (M,N,O,P,Q,R) and Severe Diabetic Pregnant (S,T,U,V,W,X) groups.

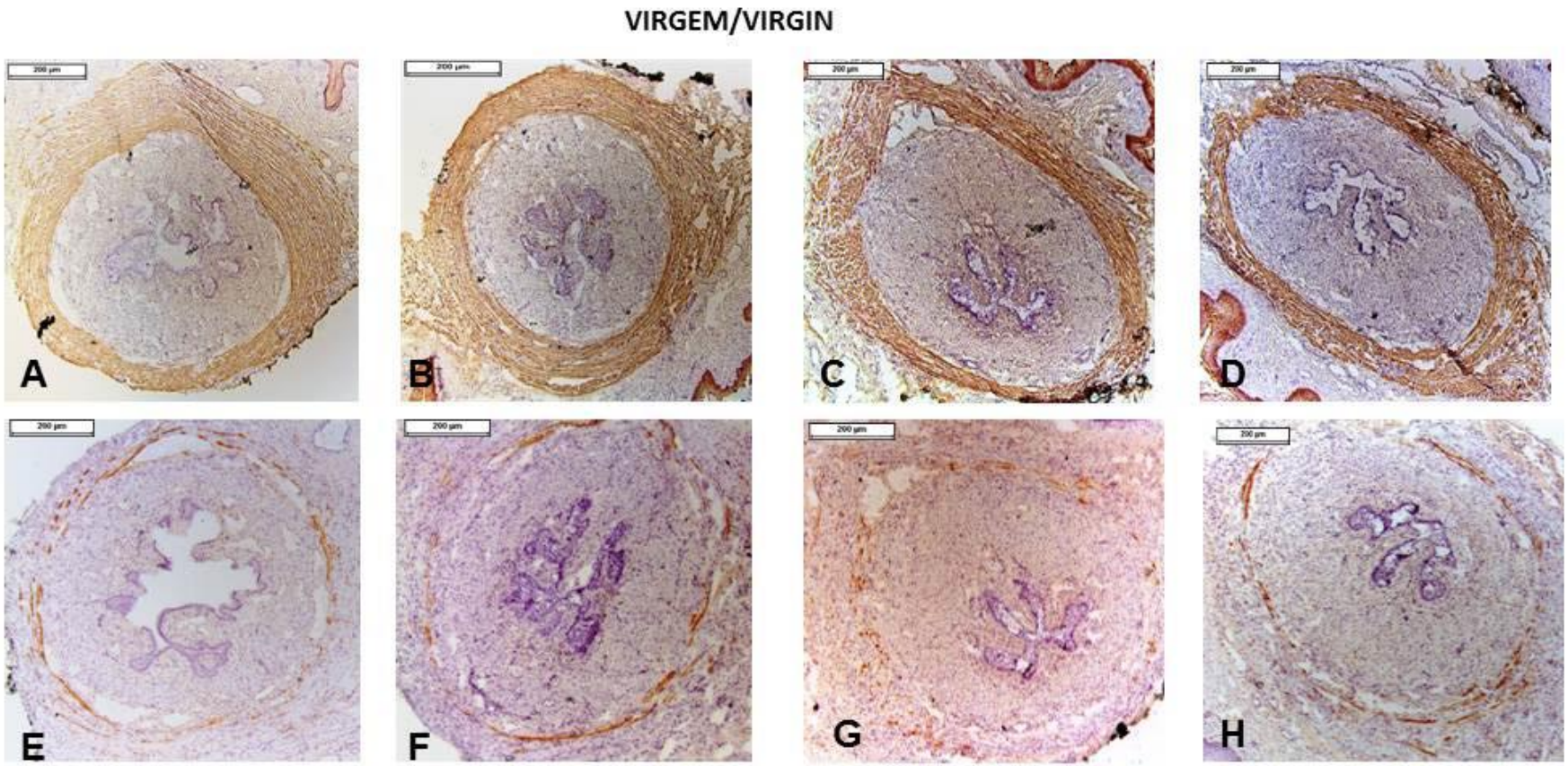

Figura 8. Imagens de cortes transversais das uretras de ratas do grupo Virgem, com imunoistoquímica para fibras rápidas (A,B,C,D) e lentas (E,F,G,H).

Figure 8. Transverse section of urethra by Immunohistochemical staining to fast (A,B,C,D) and slow fibers (E,F,G,H) in Virgin group.

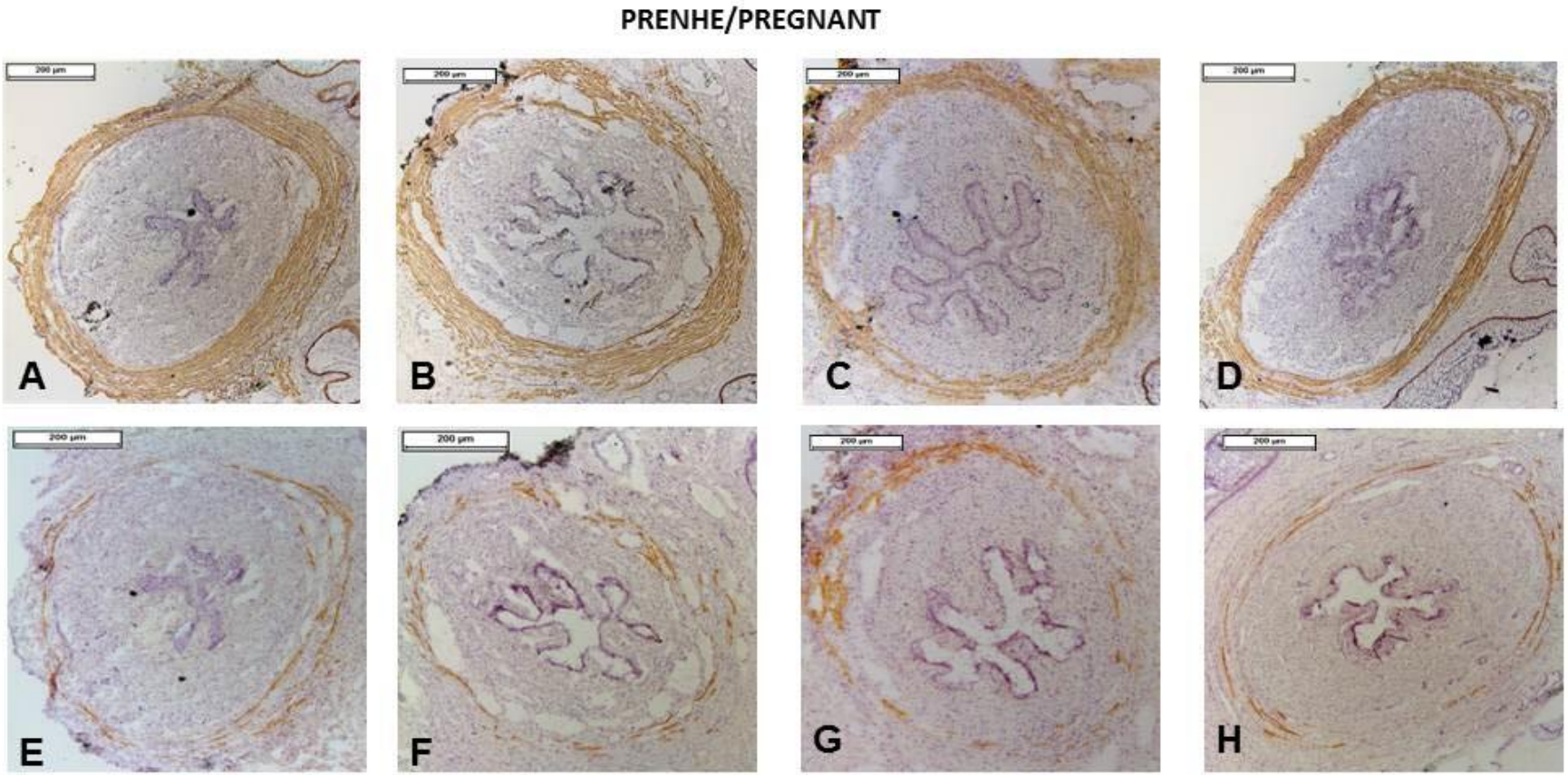

Figura 9. Imagens de cortes transversais das uretras de ratas do grupo Prenhe, com imunoistoquímica para fibras rápidas (A,B,C,D) e lentas (E,F,G,H).

Figure 9. Transverse section of urethra by Immunohistochemical staining to fast (A,B,C,D) and slow fibers (E,F,G,H) in Pregnant group.

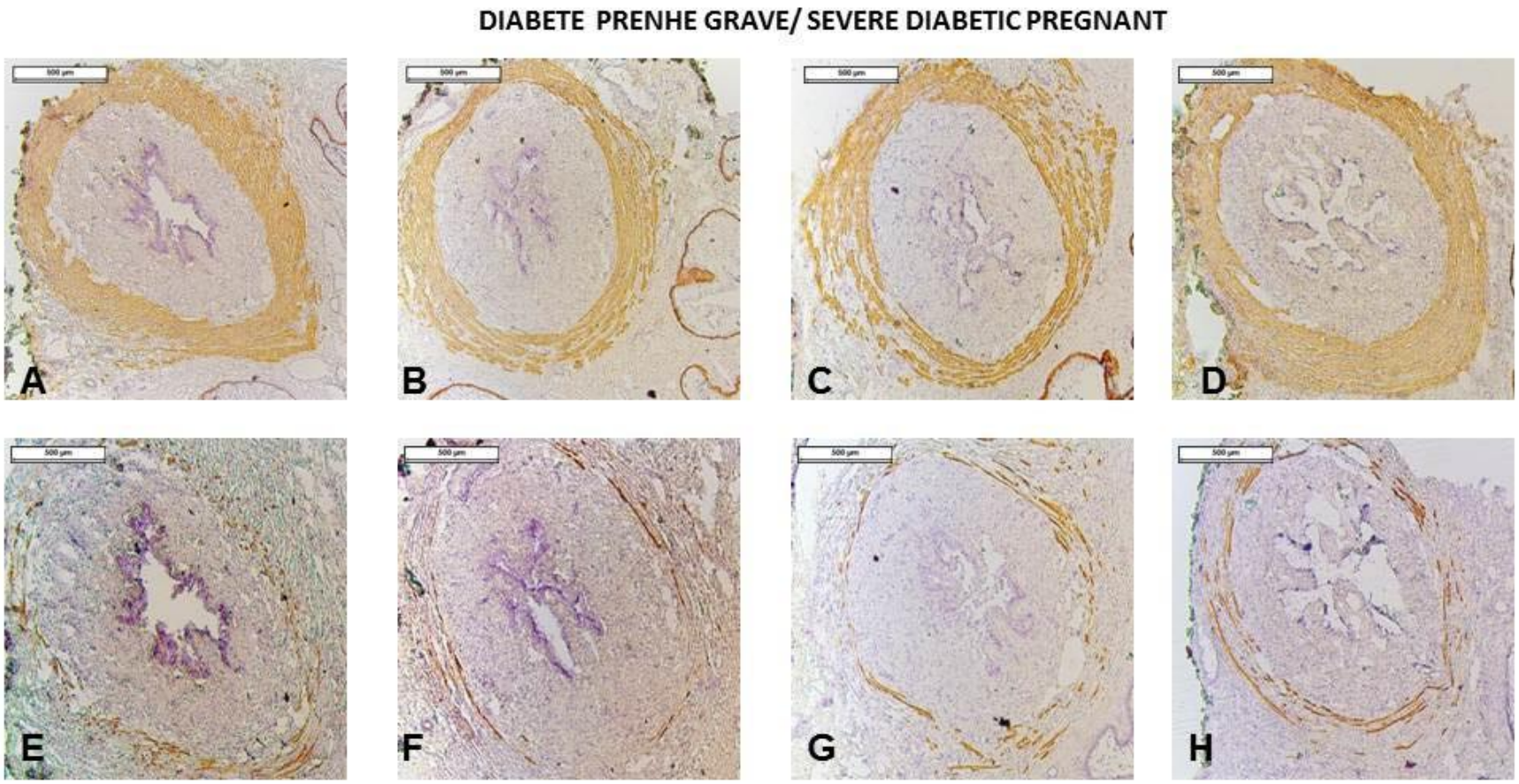

Figura 13. Imagens de cortes transversais das uretras de ratas do grupo Diabético Grave Prenhe, com imunoistoquímica para fibras rápidas (A,B,C,D) e lentas (E,F,G,H).

Figure 13. Transverse section of urethra by Immunohistochemical staining to fast (A,B,C,D) and slow fibers (E,F,G,H) in Severe Diabetic Pregnant group.

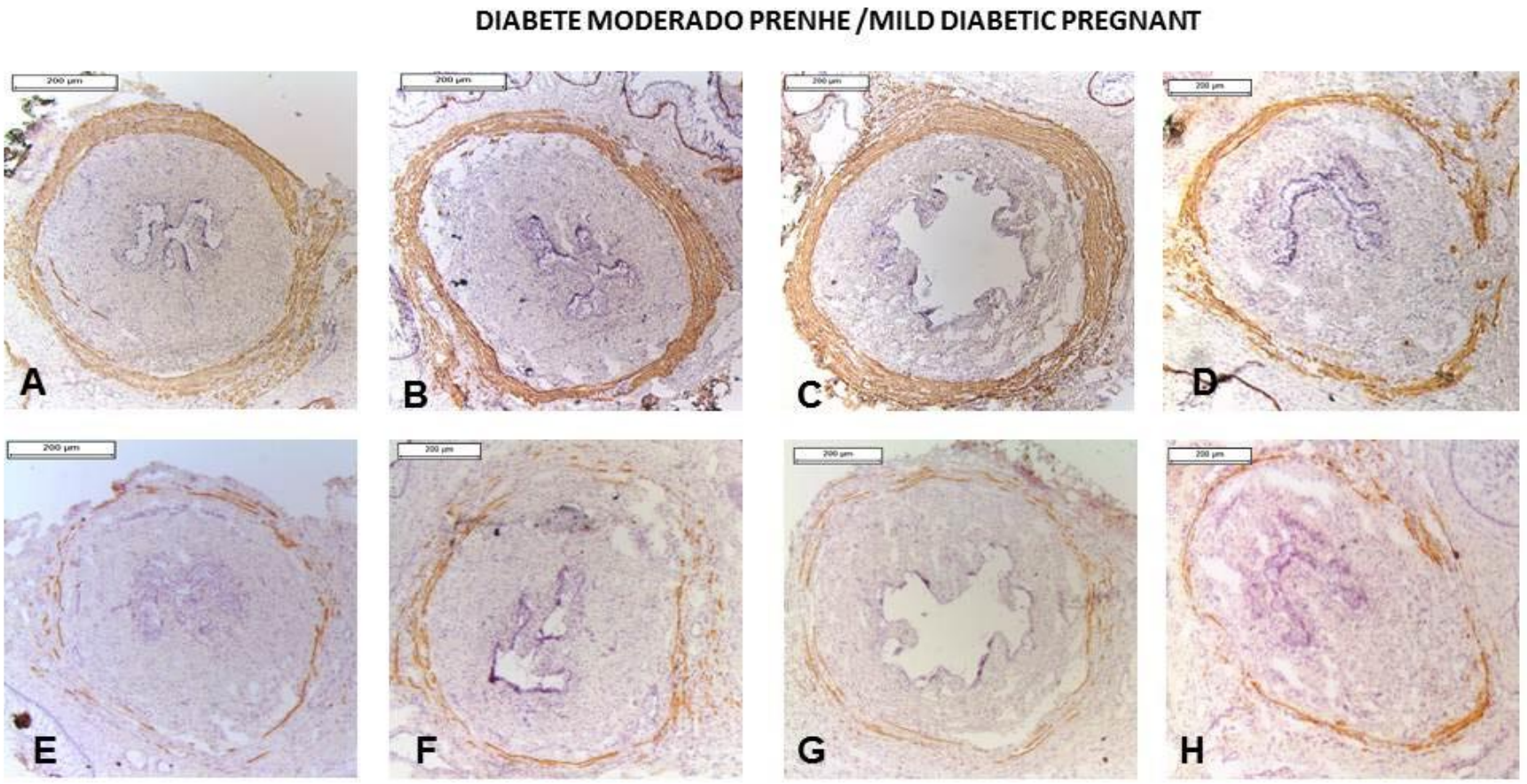

Figura 11. Imagens de cortes transversais das uretras de ratas do grupo Diabético Moderado Prenhe, com imunoistoquímica para fibras rápidas (A,B,C,D) e lentas (E,F,G,H).

Figure 11. Transverse section of urethra by Immunohistochemical staining to fast (A,B,C,D) and slow fibers (E,F,G,H) in Mild Diabetic Pregnant group.

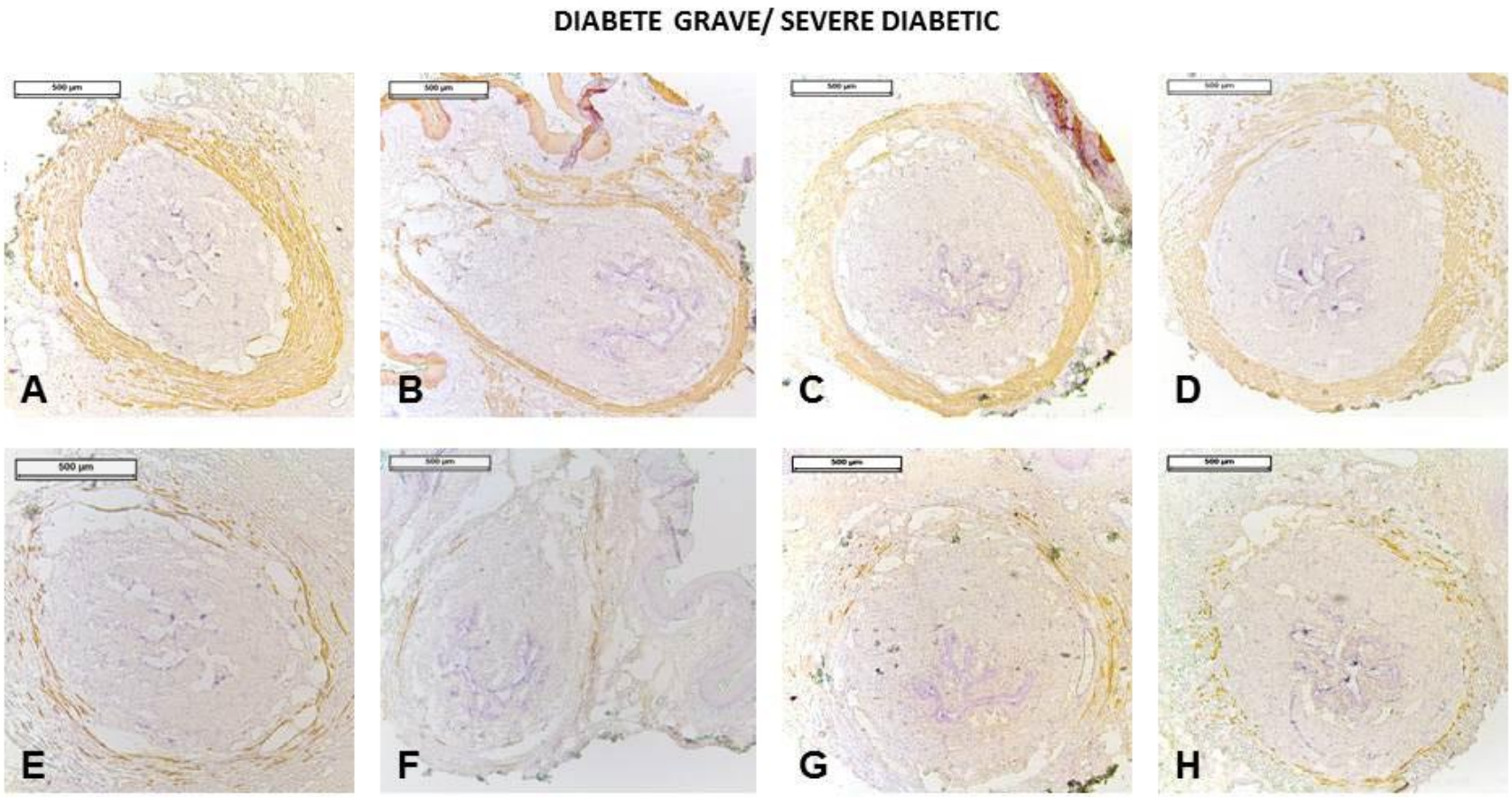

Figura 12. Imagens de cortes transversais das uretras de ratas do grupo Diabético Grave, com imunoistoquímica para fibras rápidas (A,B,C,D) e lentas (E,F,G,H).

Figure 12. Transverse section of urethra by Immunohistochemical staining to fast (A,B,C,D) and slow fibers (E,F,G,H) in Severe Diabetic group.

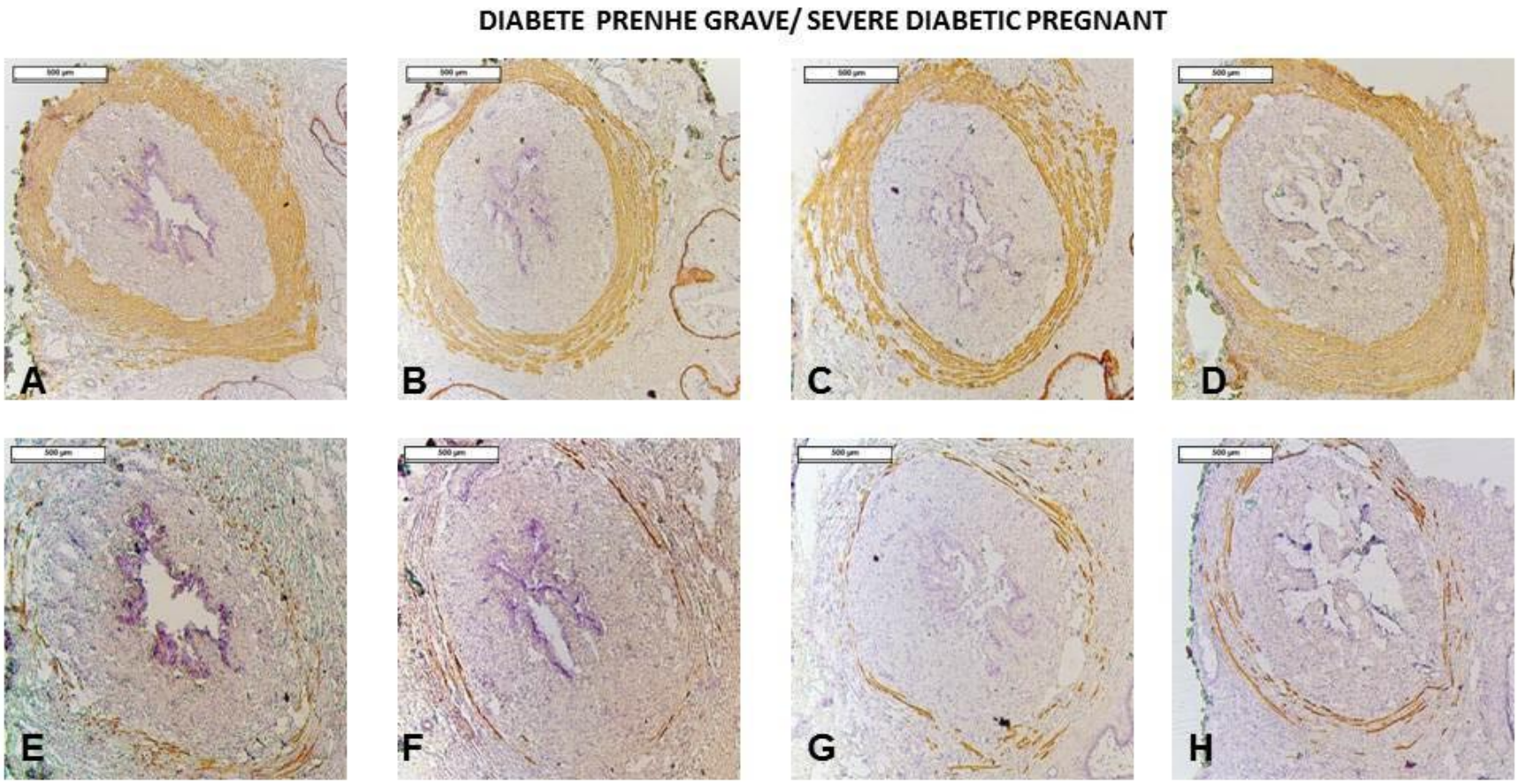

Figura 13. Imagens de cortes transversais das uretras de ratas do grupo Diabético Grave Prenhe, com imunoistoquímica para fibras rápidas (A,B,C,D) e lentas (E,F,G,H).

Figure 13. Transverse section of urethra by Immunohistochemical staining to fast (A,B,C,D) and slow fibers (E,F,G,H) in Severe Diabetic Pregnant group.

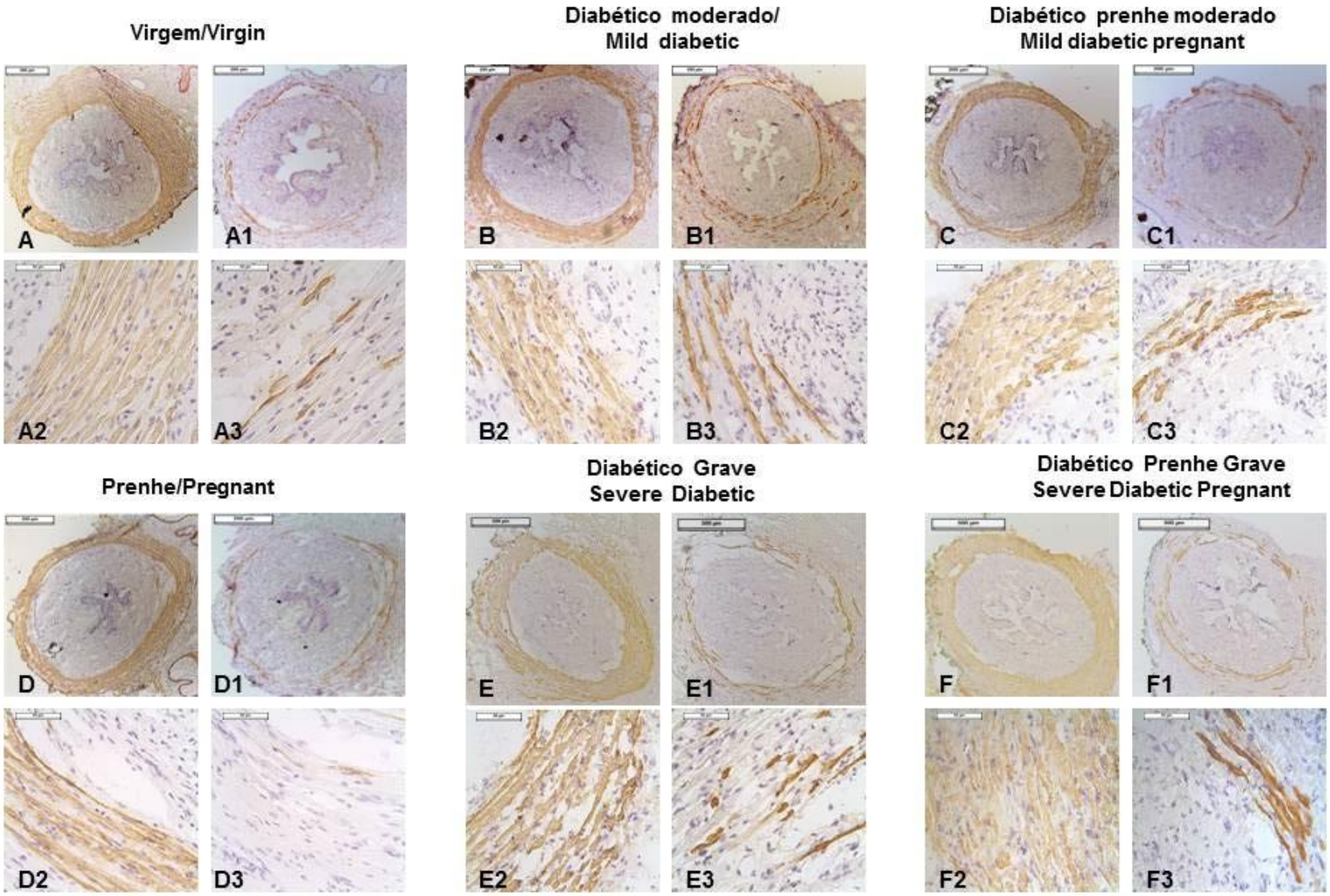

Figura 14. Imagens de cortes transversais das uretras com imunoistoquímica para fibras rápidas (A, A2, B, B2, C, C2, D, D2, E, E2, F, F2) e lentas (A1, A3, B1, B3, C1, C3, D1, D3, E1, E3, F1, F3) em todos grupos. **Figure 14.** Transverse section of urethra by Immunohistochemical staining to fast (A, A2, B, B2, C, C2, D, D2, E, E2, F, F2) and slow (A1, A3, B1, B3, C1, C3, D1, D3, E1, E3, F1, F3) fibers in all groups.

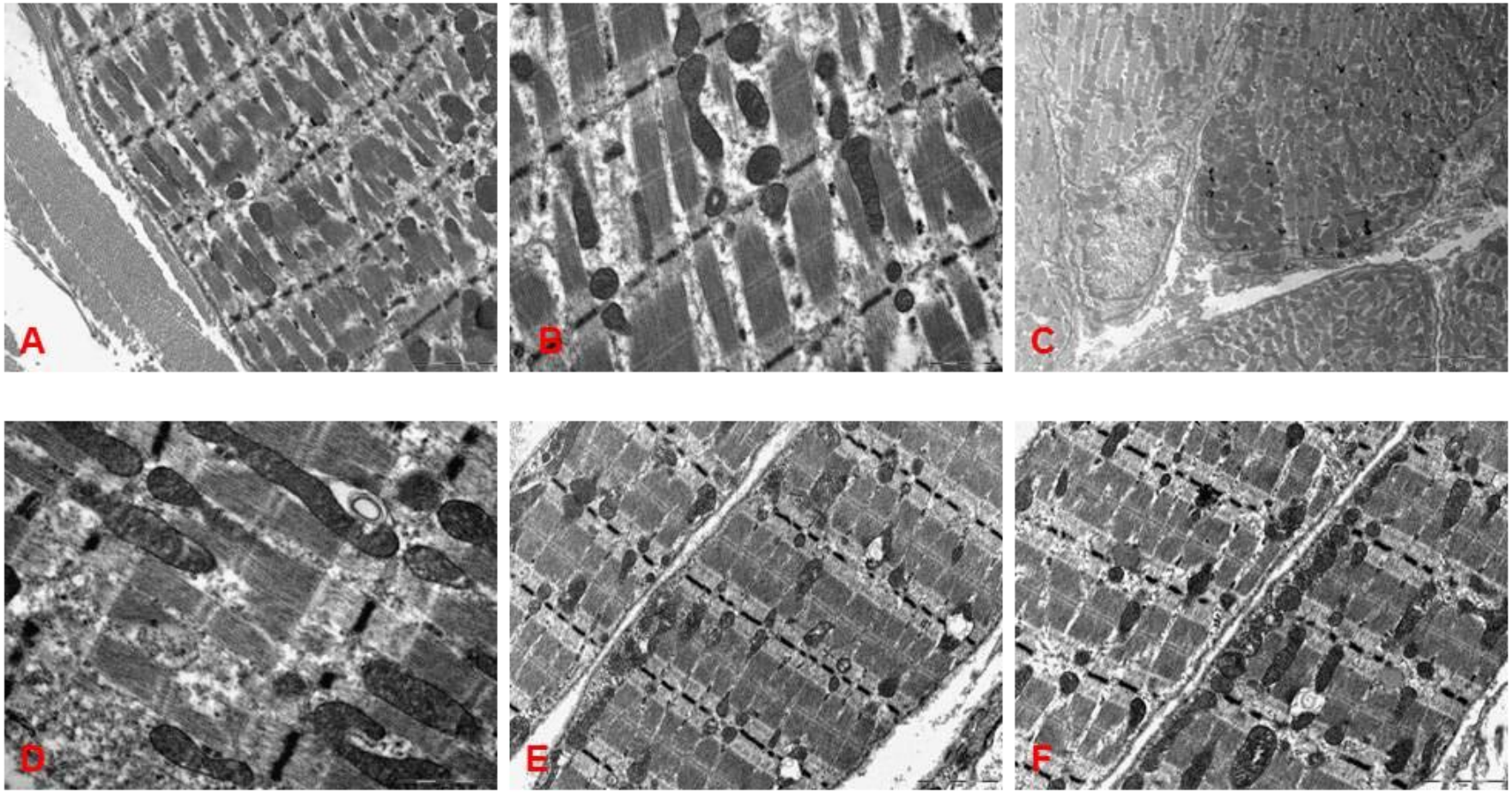

Figura 15. Imagens de microscopia eletrônica do músculo estriado uretral de ratas do grupo Virgem (A,B,C) e Prenhe (D,E,F).

Figure 15. Electron microscopy of urethral striated muscle in Virgin (A,B,C) and Pregnant groups (D,E,F).

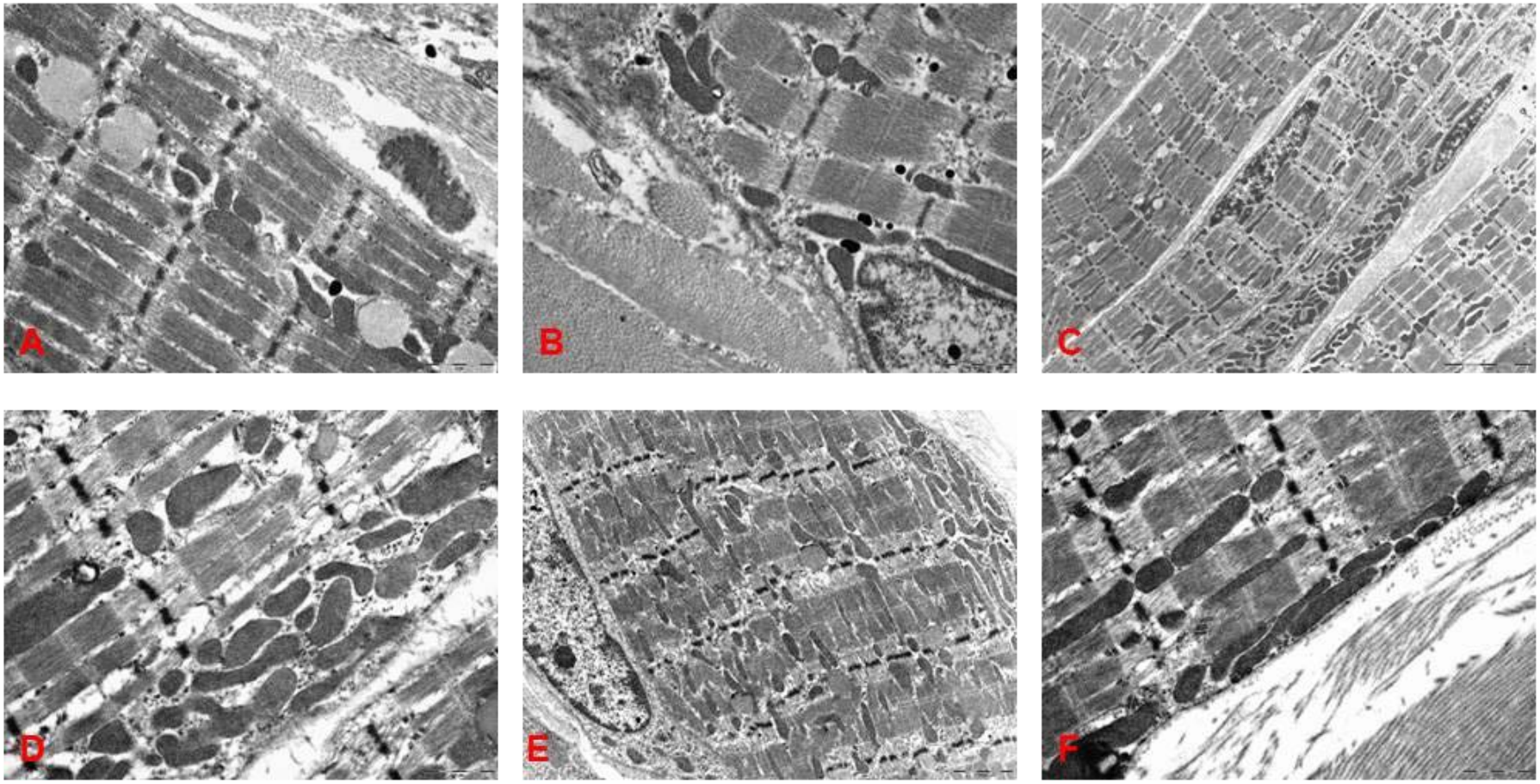

Figura 16. Imagens de microscopia eletrônica do músculo estriado uretral de ratas do grupo Diabético moderado (A,B,C) e Diabético Moderado Prenhe (D,E,F).

Figure 16. Electron microscopy of urethral striated muscle in Mild Diabetic (A,B,C) and Mild Diabetic Pregnant groups(D,E,F).

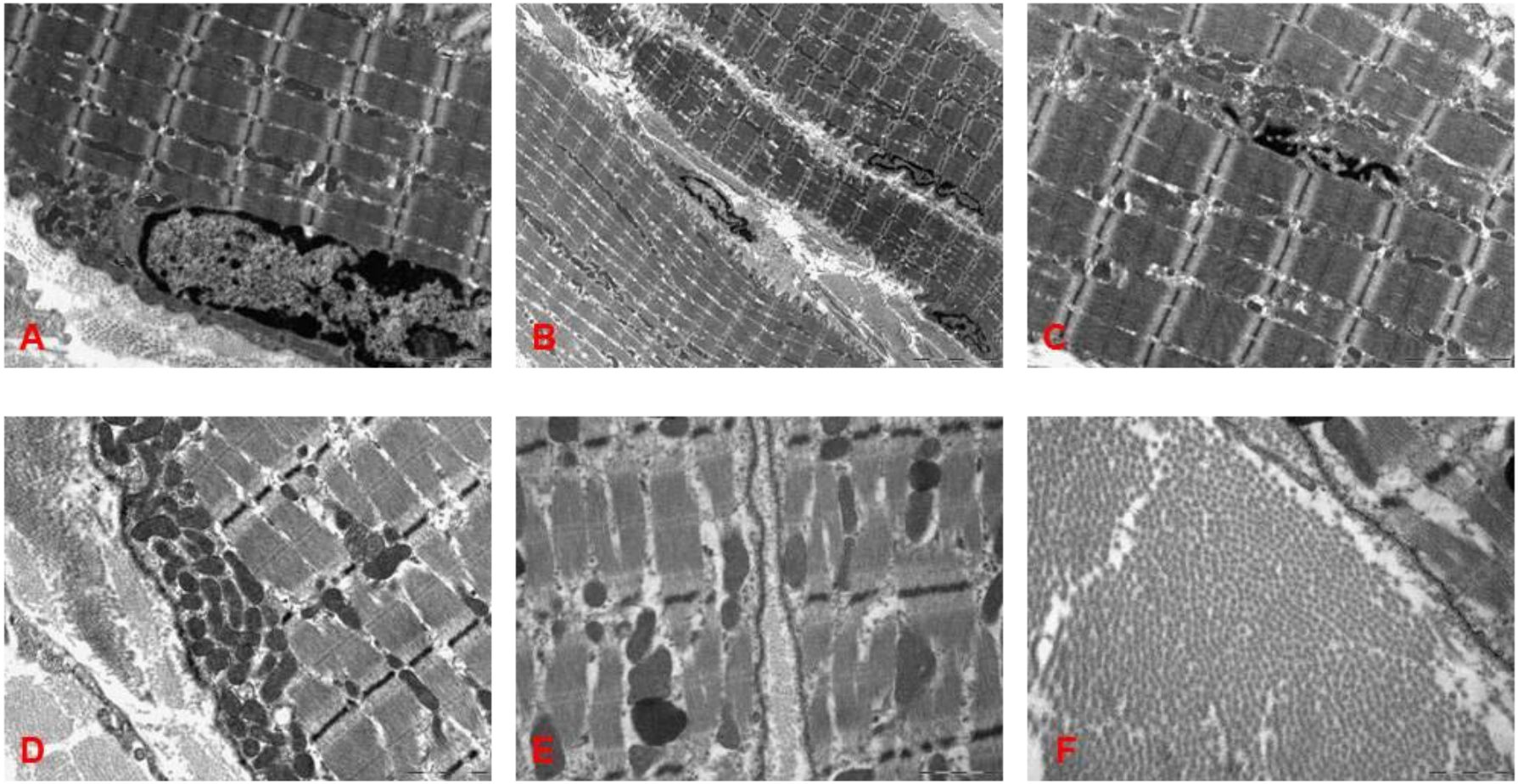

Figura 17. Imagens de microscopia eletrônica do músculo estriado uretral de ratas do grupo Diabético Grave (A,B,C) e Diabético Grave Prenhe (D,E,F).

Figure 17. Electron microscopy of urethral striated muscle in Severe Diabetic (A,B,C) and Severe Diabetic Pregnant groups(D,E,F).

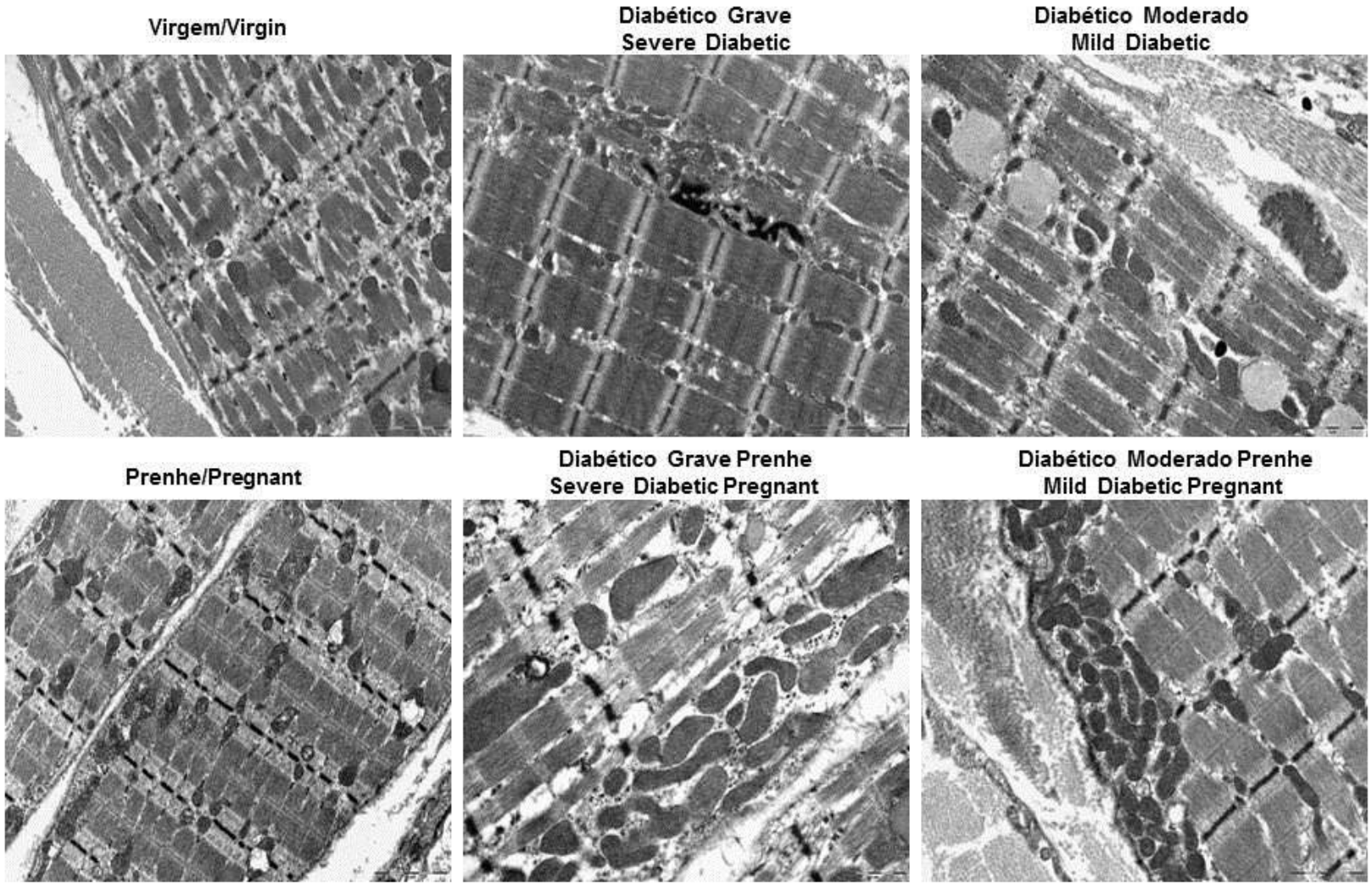

Figura 18. Imagens de microscopia eletrônica do músculo estriado uretral de ratas em todos os grupos.

Figure 18. Electron microscopy of urethral striated muscle in all groups.

Anexos

ANEXO 1 – Aprovação do Comitê de Ética

unesp

UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

FACULDADE DE MEDICINA FMB 1963 BOTUCATU

CEEA Ética

Comissão de Ética em Experimentação Animal

Criada através da Portaria DFM nº 30 de 28/04/99

Certificado

Certificamos que o Protocolo CEEA **828-2010** sobre o projeto de pesquisa "Efeito do diabete induzido por streptozotocin na matriz extracelular e no músculo estriado uretral em ratas prenhes", a ser conduzido por Gabriela Marini, orientada pela Profª. Titular Marilza Vieira Cunha Rudge, Co-orientada por Angélica Mércia Pascon Barbosa, com a colaboração de Débora Cristina Damasceno, Fernando Piculo, Jair de Campos Soares, Selma Maria Michelin Matheus e Sérgio Luis Felisbino, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), com a ressalva de que os "ratas", são provenientes de Biotério convencional, sem condições de atestar a Sanidade dos mesmos.

Projeto de Pesquisa aprovado em reunião da CEEA em 29/07/2010.

Profª Drª Regina H. Garcia Martins
Presidente da CEEA

Alberto Santos Capelluppi
Secretário da CEEA

Comissão Ética em Exp. Animal
Fls.nº 30

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